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Nitrogen-Excretion in *Carcinides maenas* (Pennant) during the Early Stages of Regeneration

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INTRODUCTION

An increased excretion of nitrogen (hereafter referred to as N) by mammals in the early stages of wound-healing raises interesting problems (Cuthbertson, 1942, 1946; Needham, 1952). The first object of the present experiments was to ascertain if the phenomena associated with this N-'flow' (Cuthbertson, 1942, 1946) occur also in *Carcinides*, a member of a very different group of animals. In mammals N-flow is greater than can be accounted for merely by the demolition of tissues damaged by the wound or by subsequent infection (Cuthbertson, 1942, 1946): the nitrogen/sulphur ratio in the urine indicates that after skin-wounds or burns much of the N comes from muscle-protein, and therefore that flow is a systematic, not a local process. It might be concerned with the provision of protein for the regeneration process itself, rather than with demolition. A Crustacean such as *Carcinides* provides critical conditions for testing these two alternatives.

By amputating the walking legs at the special autotomy-plane considerable loss may be inflicted, for subsequent regeneration, without significant damage to any tissues remaining. The response to this type of amputation may be compared with that following amputation elsewhere in the limb, removing various fractions of the limb, and causing a varying degree of damage to the tissues remaining. In these experiments it has been compared also with that following amputation of the eyes, which have no autotomy-plane, and poor powers of regeneration, and with that following excision of other structures, including portions of the body-wall. Variations in N-excretion in the early stages of wound-healing in mammals may be associated with 'shock' phenomena (Selye, 1948, 1950; Cuthbertson, 1942), due to the 'stress' of trauma, and this also may be investigated in *Carcinides*, the N-excretion of which does seem to be sensitive to 'stressing' stimuli (Needham, 1955). *Carcinides* also provides the following useful contrasts to the mammal: it is aquatic and poikolithermic, it has high

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powers of regeneration, and its exoskeleton imposes different conditions for wounding and for regeneration. Its endocrine systems are now sufficiently well known for preliminary comparisons with those of the vertebrates (Carlisle, 1954).

METHODS

The animals were kept as described previously (Needham, 1955) and their N-output measured daily over a period, before and after operation. For routine purposes the Sørensen method of estimation has been used, monitored by Kjeldahl-estimations of total N, where necessary, and the N-output is expressed throughout as its equivalent in ml. of N/100 ammonia (Needham, 1955). It has been shown (Needham, 1955) that the Sørensen-nitrogen (SN) is normally 86 per cent. of the Kjeldahl-nitrogen (KN). Output was measured for 15 days after amputation, covering the critical periods of demolition and early blastema-formation (Needham, 1952) in a crab of average size, i.e. the periods with which a 'flow'-phenomenon might be associated. Strict uniformity of body-size has not been ensured and it is probable that some consequent variation in the time-scales of the individuals has increased the variance of the results. However, this should increase confidence in those features of the responses which can be shown to be statistically significant, notwithstanding. The nature of each type of amputation is described in the appropriate section. Operations in each series were 'staggered' so as to avoid spurious patterns in the results due to weekly or other routines.

For convenience the mean SN-output just prior to operation is called the 'normal' output for that series of animals. It is assumed that, but for the experimental treatment, output would have remained constant throughout, and it is legitimate therefore to compare the mean output in any series at any time with the normal output for that series, and to apply simple tests of significance to the mean difference in output. This also provides an indirect test for the significance of differences between outputs on different days. In day-by-day records of the type obtained in these experiments, some degree of autoregression—of positive or negative serial correlation between the values obtained—may be anticipated, and significant changes in output from day to day could be due to this alone. There are methods (Kendall, 1951; Sholl, 1954) which help to distinguish between such fluctuations due to autoregression and those due to significant extraneous factors. The present records of mean output in a group of individuals are not time-series of the same type as, for instance, those showing the annual yield of corn, or the size of a population over a period of years, for which there is a unique value at each point of time, so that an estimate of variance is difficult. Here each record is the average of a number of individual time-series, and the sampling variance of each mean value may be estimated. The significance of the difference between any two means may be tested in the usual way—always remembering that the significance revealed may be that of autoregressive effects, or of extraneous factors, or both.

TABLE 1

Summary of results

Mean daily output of SN per individual, as ml. of N/100 NH_3.

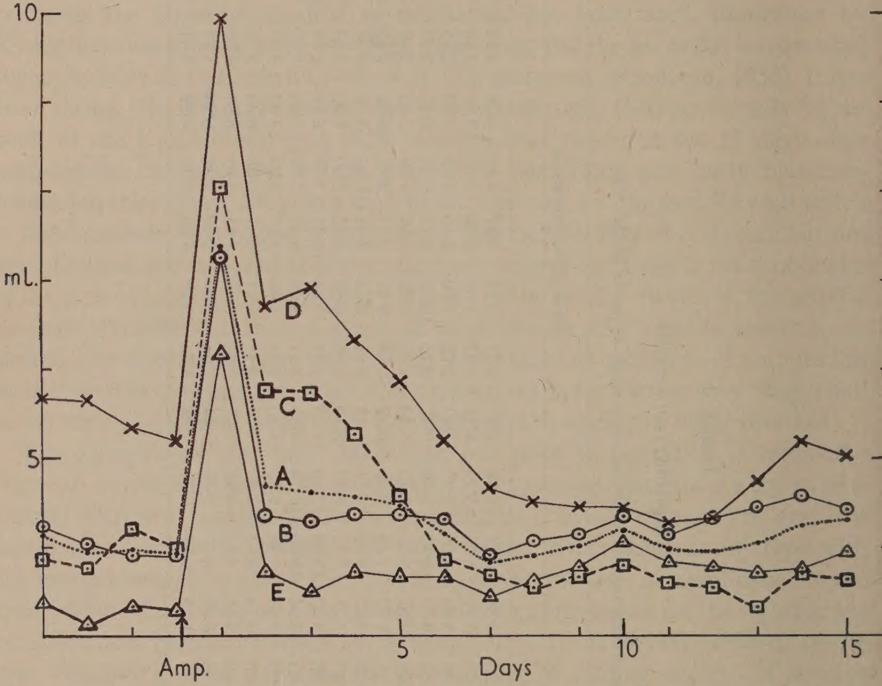
Series	Number of records	*Days, pre-amputation*				*Days, post-amputation*														
		4	*3*	*2*	*1*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
1.1	95	4·24	4·03	3·92	3·91	7·26	4·38	4·29	4·38	4·37	4·31	3·92	4·07	4·15	4·36	4·14	4·32	4·45	4·58	4·41
1.2	32	3·88	3·77	4·20	3·98	8·03	5·77	5·73	5·28	4·58	3·87	3·69	3·56	3·68	3·81	3·60	3·54	3·32	3·71	3·63
2·1	23	3·49	3·31	3·60	3·41	8·96	5·64	4·06	2·87	2·93	2·62	3·00	3·10	3·15	2·83	2·85	2·52	2·07	2·13	2·28
2·2	42	9·06	9·73	8·60	8·52	13·63	9·77	8·85	8·36	9·82	9·03	10·52	9·11	9·41	9·83	9·83	9·57	9·19	9·42	10·40
3.1	53	4·30	4·28	4·57	4·27	9·09	4·62	5·31	5·25	5·40	4·73	4·92	4·89	5·29	5·62	5·40	5·44	5·27	5·08	4·55
3.2	21	8·95	7·96	8·59	7·69	12·21	9·72	11·23	8·56	10·32	10·08	9·41	9·37	8·45	8·52	8·52	8·36	7·06	7·57	7·62
4	23	10·23	10·75	9·21	9·28	11·70	10·62	8·77	9·34	10·72	8·48	9·24	8·95	9·55	8·78	9·24	8·54	8·35	9·09	9·13
5.1	29	5·59	5·09	5·59	5·79	9·47	7·21	7·15	6·57	6·34	6·95	6·90	6·52	6·99	5·92	5·62	5·90	5·26	5·63	5·96
5.2	24	5·33	4·95	5·52	4·78	16·70	15·97	13·08	12·20	11·53	11·23	9·08	10·07	7·58	6·88	6·38	6·54	6·33	6·03	6·08
5.3	23	4·21	3·90	3·66	3·91	15·43	12·96	10·06	10·74	8·58	8·11	8·40	8·45	7·99	7·63	7·15	7·11	7·09	6·86	6·41
5.41	11	4·70	4·76	5·27	5·37	7·75	4·03	5·38	4·51	4·10	3·48	3·46	3·87	3·37	3·43	3·37	3·26	3·30	3·27	3·85
5.42	7	5·41	5·16	5·66	4·57	14·60	10·78	9·63	8·53	7·56	8·15	6·81	6·87	6·86	6·44	5·36	5·56	4·49	4·00	3·83
6	17	6·05	5·81	5·41	5·74	7·60	5·08	4·83	4·94	5·37	5·21	5·43	5·45	5·45	6·01	5·48	5·74	5·81	5·57	5·27
7.1	17	8·71	7·14	6·80	6·71	7·55	6·92	6·77	7·48	7·44	7·95	7·94	8·00	7·36	6·94	5·78	5·55	5·24	4·93	5·33
7·2	8	6·81	6·46	5·88	6·34	10·74	11·23	9·88	9·33	7·38	5·78	5·00	5·00	4·36	4·99	4·41	4·14	4·84	4·66	5·68

RESULTS

The results as a whole are summarized in Table 1.

1. *Walking-legs: amputation at the autotomy-plane, in fasting individuals* (127 crabs, 41 in isolation)

The mean daily SN-output for 127 individuals over the 4 days just prior to



TEXT-FIG. 1. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Amputation of one pair of walking-legs at the autotomy-plane in fasting individuals: *A*, all results, pooled (127 crabs); *B*, first pair of limbs only (95 crabs); *C*, second pair of limbs only (32 crabs); *D*, isolated individuals only (41 crabs); *E*, grouped individuals only (86 crabs).

amputation was equivalent to 3·96 ml. of N/100 NH_3 per individual (Text-fig. 1A). This was lower than that previously recorded for fasting crabs (Needham, 1955), mainly because of the smaller average body-size. During the first day after amputation output increased to 7·39 ml. per individual, i.e. very nearly double the normal output. KN increased relatively more than SN. During the second day output fell to a value about 16 per cent. above normal, and the curve of output/time inflected sharply at this point, marking the onset of a slower decline in output, to reach a minimum, at the normal value, on the seventh day. Output then rose slightly but progressively, to a low peak on the tenth day, declined somewhat to a second minimum, and subsequently increased progressively until the end of the period.

These fluctuations were small in amplitude but there were significantly few turning-points and in view of the large size of the sample it seems reasonable to recognize as significant four phases in the record: (1) a steep climb in output on the first day followed by a steep decline on the second, (2) a 5-day period of slower decline to the normal value, (3) a 5-day cycle of higher output, (4) a final increase in output.

The 127 records of this series included 32 (series 1.2) in which a second pair of limbs was amputated, 16–20 days after the first operation. On separating these from the remainder (series 1.1), both subgroups gave mean records (Text-fig. 1 B, C) showing the same essential features. The only important difference was that the initial increase in output of phase 1 was greater after the second amputations, and remained higher during the first part of phase 2. Later, the decline was more rapid and the general level of output lower than in series 1.1. The times of some of the turning-points differed slightly.

A number of the animals of this series were kept in groups of 2–10, and the mean output of the group measured. On plotting separately the results for groups and for isolated individuals there appeared to be significant differences in the character of the response (Text-fig. 1 D, E). The low normal output of this series may have been due partly to grouping (Needham, 1955), and after amputation there appeared to be also a differential depression of output in phase 2 as contrasted with that in phase 3. Indeed, the raised output in phase 3, in the pooled results, appeared to be due almost entirely to the grouped individuals (Text-fig. 1 A, E). For grouped individuals, mean output on the tenth day was significantly higher than on the seventh day (Student's $t=2{\cdot}92$, $n=35$).[1] For isolated individuals, by contrast, output on the third day was significantly higher than normal ($t=3{\cdot}13$, $n=39$), and the decline between the third and eighth days was significant ($t=3{\cdot}81$). None of these features was significant in the other subseries or in the pooled results. Ignoring the phase 1 peak the general trend in output of grouped crabs was upwards while that of isolated individuals was downwards—until the final phase. For isolated individuals the record between days 1 and 12 approximated to what, for convenience, may be termed an 'exponential' curve, i.e. the rate of decline itself declining progressively.

The methods of Kendall (1951), as applied by Sholl (1954), indicated a periodicity of 6·2 days in the graph of mean output for all individuals of the first experiment, assuming that there was no basic trend. This value is consistent with the periodicity indicated by the graphs. The oscillations of the 'correlogram' showed no evidence of damping, so that the cycles of the output-record were probably due to extraneous causes, associated with the injury.

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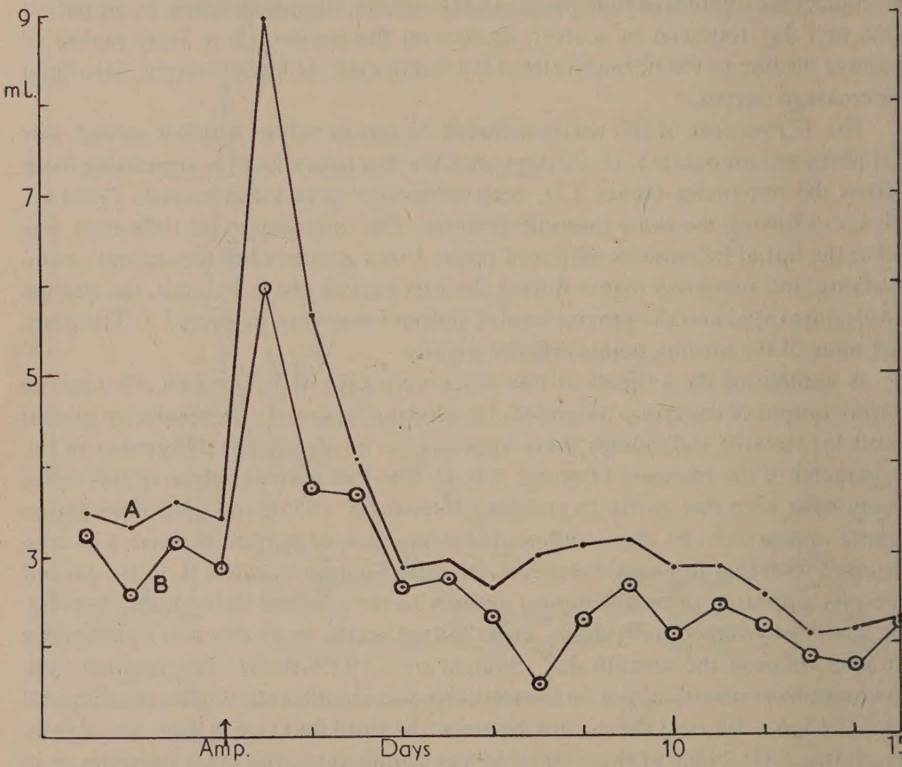
[1] n=number of groups, here and elsewhere.

2. *Walking-legs: amputation at the autotomy-plane in feeding individuals*

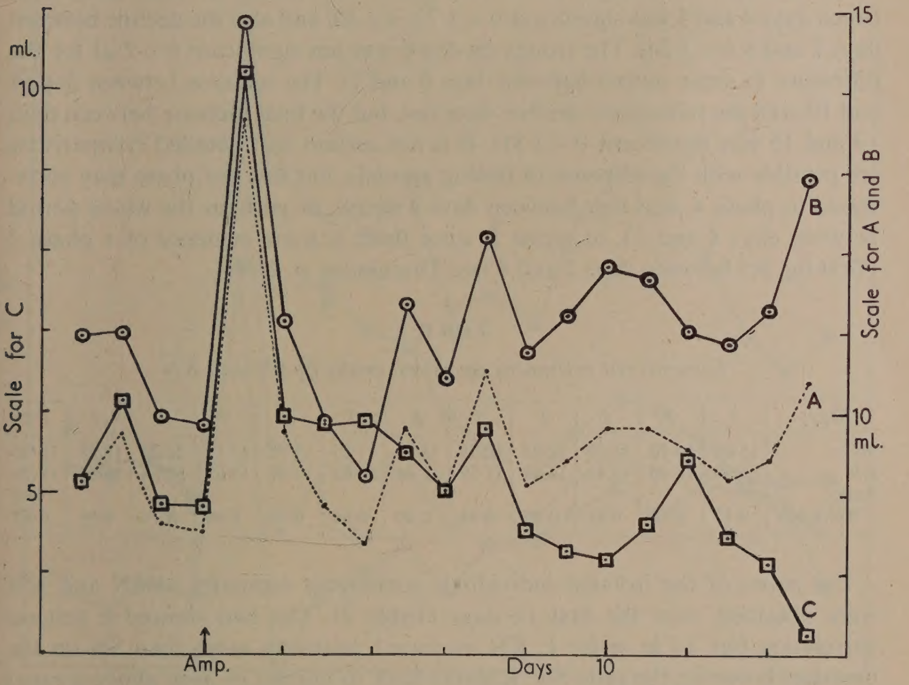
2.1. *High carbohydrate diet* (23 crabs, 13 isolated). Crabs were fed on bread until output was steady (Needham, 1955) before operation. The normal mean output on a bread diet is relatively low (Needham, 1955). However, the initial increase was greater (Table 1; Text-fig. 2A) than in fasting individuals, and the subsequent steep phase of decline more prolonged, so that there was no inflexion on the second day in the graph of mean output/time and therefore no distinction into phases 1 and 2. Output was still significantly above normal on the second day ($t = 3{\cdot}16$, $n = 14$). From the fourth day onwards output was consistently below normal in contrast to that in series 1, though there was a later cycle of relatively raised output corresponding in time to phase 3 of series 1, and an indication of a final rise in output. However, the increase between days 7 and 9 was not statistically significant. The generally low output during the later part



TEXT-FIG. 2. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Amputation of one pair of walking-legs at the autotomy-plane in crabs feeding on bread: *A*, all results, pooled (23 crabs); *B*, isolated individuals only (13 crabs).

of the period appeared to be significant; mean output for all twenty-three was significantly below normal on the 13th day ($t = 3{\cdot}08$, $n = 14$). For isolated individuals, alone, output was already significantly below normal on the seventh day ($t = 4{\cdot}23$, $n = 12$).

The record for isolated crabs only (Text-fig. 2B) showed essentially the same features, allowing for the irregularities of a small sample, the major trough on day 7 marking the transition between phases 2 and 3. The transition between phases 1 and 2 was somewhat more definite than in the record for the whole series.



TEXT-FIG. 3. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Amputation of one pair of walking-legs at the autotomy-plane, in crabs feeding on earthworm: *A*, all results pooled (42 crabs); *B*, isolated individuals only (31 crabs); *C*, grouped individuals only (11 crabs).

2.2. *High protein diet* (42 crabs, 31 in isolation). For this purpose crabs were fed 1·0 per cent. of their body-weight of fresh earthworm per day (Needham, 1955), more than adequate for N-equilibrium under normal conditions. The mean daily output before operation was about twice that in series 1 (Table 1), but the increase on the first day after amputation was not much greater than in that series. The subsequent decline was steeply and smoothly graded to a minimum just below normal on the fourth day (Text-fig. 3A) as in series 2.1.

Subsequently there was a generally upward trend in mean output, in isolated individuals (Text-fig. 3B), and the curve differed from that for series 1 and 2.1 also in the more violent short-term fluctuations in output. The number of grouped individuals was small but their record (Text-fig. 3C) indicates a generally downward trend in output. As in series 1 therefore the trend was opposite to that for isolated individuals and both trends were opposite to that of the counterpart in series 1.

In the record for isolated protein-fed crabs, the increase in mean output between days 4 and 5 was significant ($t=3{\cdot}73$, $n=30$) and also the decline between days 7 and 8 ($t=3{\cdot}24$). The trough on day 6 was not significant ($t=2{\cdot}21$ for the difference in mean output between days 6 and 7). The increase between days 8 and 10, and the subsequent decline were not, but the final increase between days 13 and 15 was significant ($t=2{\cdot}81$). It is not certain that detailed comparisons are possible with the response of fasting animals, but this last phase may correspond to phase 4, and that between days 4 and 8, or perhaps the whole period between days 4 and 13, to phase 3, since there is some evidence of a phase 2 (Text-fig. 9C) between days 2 and 4 (see Discussion, p. 210).

TABLE 2

Concurrent estimates on seven crabs of SN and KN

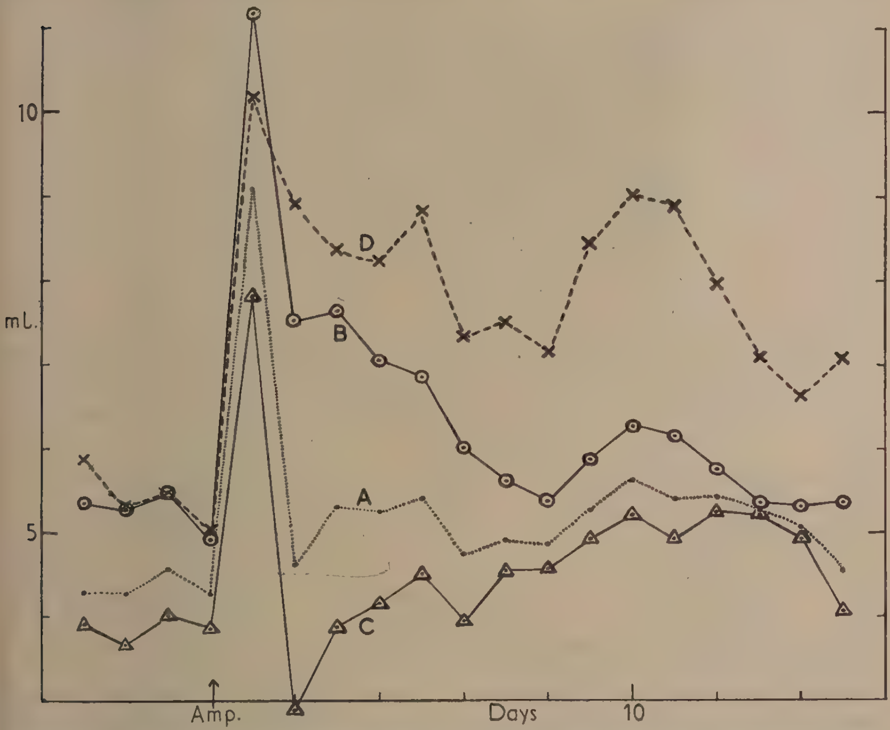
Day:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
SN	15·07	10·10	11·28	12·03	10·35	13·05	17·25	13·96	17·13	24·29	18·18	16·80
KN	28·98	11·40	12·43	14·11	11·19	14·44	20·64	17·89	19·87	30·64	19·65	18·79
Ratio SN/KN	0·52	0·89	0·91	0·85	0·93	0·90	0·84	0·79	0·86	0·79	0·93	0·89

For seven of the isolated individuals concurrent estimates of SN and KN were obtained, over the first 12 days (Table 2). The two showed a general correlation but, as in series 1, KN increased relatively more than SN on the first day; however, the ratio SN/KN was back to normal or even above normal by the second day. In the subsequent oscillations, in general, the ratio was above normal when output was low and below normal when output was high, i.e. any increase in output was due more to other components than to ammonia.

The extent of individual variations is shown by the differences between the graph of mean SN for this sample and that for all thirty-one isolated individuals. That it is a genuine sampling variation is shown by the fact that the graph of mean KN for the sample has the same form, in detail. This form indicates a phase 2 between days 2 and 5 and a long phase three, with its major peak on day 10.

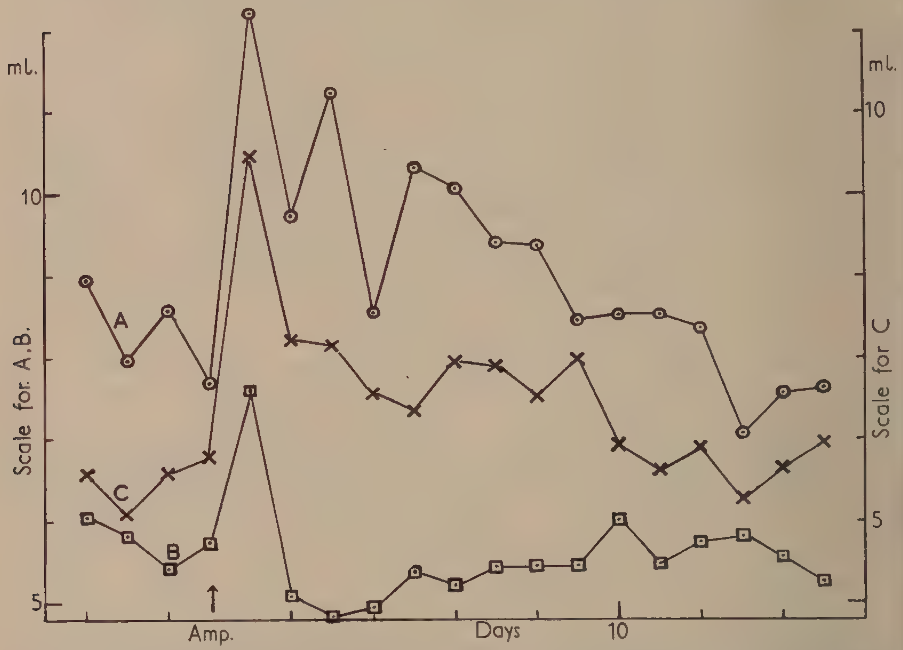
3. *Walking-legs: amputation involving damage to tissues remaining*

3.1. *Amputation proximally to the autotomy-plane* (53 fasting crabs, 33 grouped and 20 in isolation). The normal output was about the same as in series 1, but the increase on the first day after amputation was greater, and output remained relatively higher throughout (Text-fig. 4A); it was scarcely back to normal at the end of the period and there seems no doubt that the greater damage to tissues remaining caused this persisting higher output. In detail there seemed good evidence of the four phases already recognized, particularly in the records for isolated crabs (Text-fig. 4 B, D). These features, apart from the initial peak, were not statistically significant for the series as a whole, but this was partly due to differences in response by isolated and grouped individuals (Text-fig. 4 B, C), differences similar to those in series 1, and greater in magnitude. In a subgroup of ten individuals which sustained most damage to the stump, the general level of output was particularly high (Text-fig. 4D), and the decline between days 5 and 6 was significant ($t = 3{\cdot}82$, $n = 9$), while the increase between days 8 and 10 and the decrease between days 10 and 13 approached the level of significance ($t = 2{\cdot}62$ and $2{\cdot}92$ respectively).



TEXT-FIG. 4. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Amputation of one pair of walking-legs immediately proximally to the autotomy-plane: *A*, pooled results (53 crabs); *B*, isolated individuals (20 crabs); *C*, grouped individuals (33 crabs); *D*, individuals with extensive damage to the stump.

3.2. *Amputation through the distal end of the propus* (21 isolated crabs, 3 feeding and 18 fasting). Here the amount of damaged tissue left *in situ* was about equal to that in series 3.1 but the bulk of tissue removed was much less. Limbs amputated here are not frequently autotomized, as they are after amputation at more proximal levels in the limb. Here (Text-fig. 5A), as in series 3.1, mean output, while fluctuating considerably, remained considerably above normal until the ninth day, the difference being still statistically significant on the fifth day ($t = 3{\cdot}03$, $n = 20$). The general downward trend in output was more nearly linear than in previous series. The decline in output between third and fourth days and the increase between fourth and fifth were both significant ($t = 3{\cdot}53$, $4{\cdot}36$ respectively, $n = 20$). The trough on day 4 therefore probably marks the transition between a short phase 2 and a long phase 3, as in series 2.2. The other fluctuations in output were not significant, but when the results were divided into two subgroups—a feasible technique for distinguishing fortuitous from consistent features—the peaks on days 1 and 3 and the troughs on days 2, 4, and 13 were present in both. The two records differed only in details of phase 3.

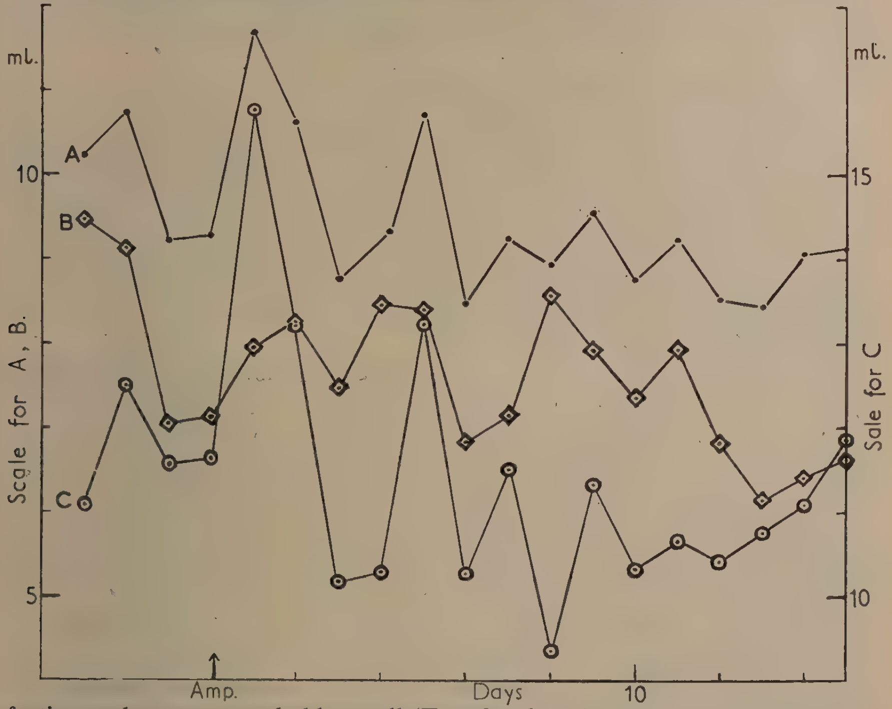


TEXT-FIG. 5. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). *A*, amputation of one pair of limbs through the propus (21 crabs); *B*, amputation of both of the second pair of antennae (17 crabs); *C*, amputation of one eye (29 crabs; curve *A* of Text-fig. 7 drawn to a different scale).

4. *Walking-legs: spontaneous autotomy* (23 isolated crabs, 11 of them feeding)

Autotomy causes less damage to tissues remaining than any surgical operation. Amputation, even at the autotomy-plane, usually results in slight damage proximally to the plane, or leaves fragments of tissue distally to it. Autotomy was induced by preliminary amputation through the distal end of the merus.

TEXT-FIG. 6. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Induction of autotomy in one pair of walking-legs: *A*, pooled results (23 crabs); *B*, fasting individuals (12 crabs); *C*, feeding individuals (11 crabs).



The increase in mean N-output on the first day after operation, particularly in fasting crabs, was remarkably small (Text-fig. 6 A, B), by comparison with that in most other series, though it was statistically significant ($t=4{\cdot}29$, $n=20$), owing to the feeding individuals (Text-fig. 6C). The subsequent decline in output to a minimum on the third day was significant ($t=2{\cdot}82$, $n=22$, for the mean difference in output between second and third days). The subsequent increase between third and fifth days was significant only at the 2 per cent. level ($t=2{\cdot}59$), but the decrease from fifth to sixth day was more clearly significant ($t=2{\cdot}83$). Subsequent oscillations were insignificant, and output was thenceforth around normal in fasting individuals, and subnormal in feeding individuals (contrast series 2.2).

Since the trough on day 3 in fasting crabs was less significant than that on day 6, it probably marks the transition between phases 1 and 2, and that on day 6 the transition between phases 2 and 3, i.e. the time-scale corresponds more closely to that in series 1 and 3.1 than to that of the feeding individuals and of series 2.2 and 3.2. As in other series there were irregularities in output in phase 3, here due to asynchromy between the response of fasting and feeding crabs, as well as to individual variability, which probably increased with time from the date of operation. As in most other series there was a trough in output around day 13, and a final increase (phase 4).

In fasting crabs there was a marked decline in mean output during the few days before operation, but this, for instance the decline between third and second days prior to operation, was not statistically significant ($t = 1{\cdot}90$, $n = 22$).

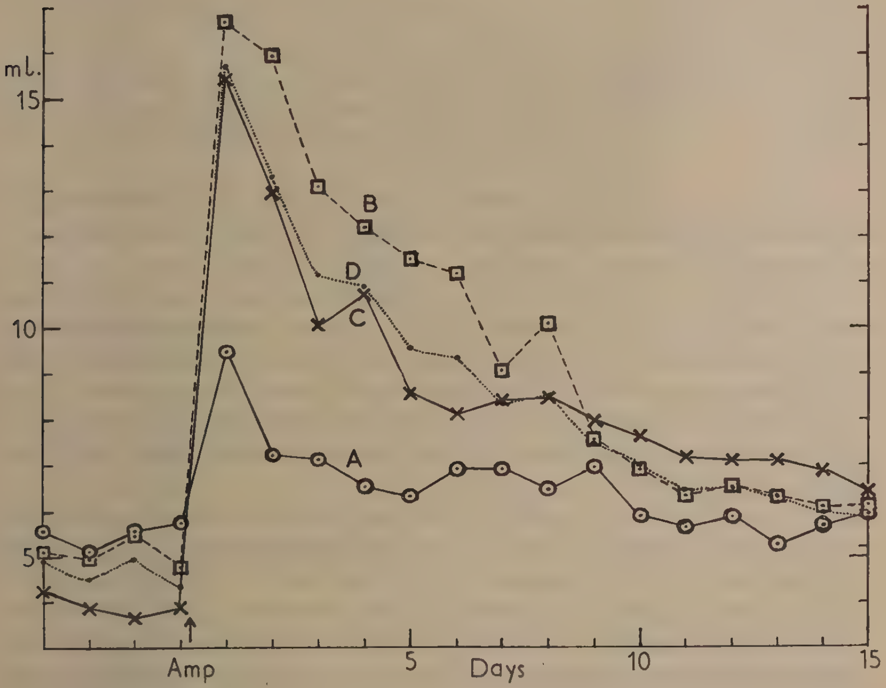
5. *Eyes: amputations through the eye-stalk*

In addition to the value of this operation for comparison with leg-amputation, a specific effect on N-metabolism of removing the eye-stalk endocrines is to be anticipated. These endocrines are known to affect growth and ecdysis (Carlisle, 1954), and therefore also regeneration, and they affect the amount of protein in the body (Nieland & Scheer, 1953; Leone, 1953). Haemorrhage was reduced by sealing the wound with celloidin solution. For convenient presentation it has been necessary to reduce the scale of N-output in this series (Text-fig. 7) to one-half of that in other series.

5.1. *Amputation of one eye, at the base of the stalk* (29 crabs, 17 fasting and 12 feeding). The increase in N-output on the first day was intermediate between those for series 1 and series 2.2, and the relative decrease on day 2 was comparable. Subsequently (Text-fig. 7A) output remained somewhat above normal, but the mean output for the days 2–8 was not significantly greater than before operation ($t = 1{\cdot}96$, $n = 28$). There was a general, if slight, downward trend in output, from the first day, throughout the period, reaching the normal level around day 13.

When these results were plotted (Text-fig. 5C) on the same scale as those of other series the graph resembled in general form those of typical records, e.g. of series 1, 2.1, 3.1, and 3.2 (Text-figs. 1 C, D; 2 A, B; 4B; 5A); i.e. following the initial peak there was a phase 2 of slow decline from days 2 to 5, followed by a phase 3 cycle of increased output between days 5 and 13, followed by the final increase in output. Phase 3 was probably more evident against the 'base line' of an overall exponential decline than against that of the normal output. These oscillations were not statistically significant, however, after day 2. The separated records for feeding individuals showed the two phases 2 and 3 very clearly but for fasting animals phase 3 was broken up and indefinite.

5.2. *Amputation of the second eye at the base of the stalk* (24 survivors of series 5.1, 12 fasting and 12 feeding). The increase in mean N-output on the first day (Table 1; Text-fig. 7B) was nearly three times that after removal of the first eye and much greater than after any other operation, so that there seems little doubt that complete removal of the endocrine complex of the eye-stalks causes a specific change in N-metabolism. Output returned towards normal, absolutely faster but relatively more slowly than in series 5.1, so that it was still above normal at the end of the period. This difference was statistically significant up to the tenth day. The curve may be regarded as roughly exponential in form, with minor oscillations superimposed.



TEXT-FIG. 7. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Amputation of eye-stalks: *A*, one eye only (29 crabs); *B*, second eye (24 crabs); *C*, both eyes concurrently (23 crabs); *D*, pooled results, second eye, both eyes and both eyes amputated in the middle of the stalk (54 crabs). The scale of NH_3 output is one-half that of the other text-figures.

5.3. *Concurrent amputation of both eyes at the base of the stalk* (23 crabs, 17 fasting and 6 feeding). The increase in mean output on the first day was virtually the same as after the removal of the second eye, in succession (Text-fig. 7C). Subsequent output also showed essentially the same exponential decline, but with a more rapid decline at first and a slower decline later, so that the excess output per day at the end of the period was somewhat greater. In this series the minor oscillations about the general exponential trend compared in time-scale with those in series 5.1, and in other series.

KN was measured in six individuals after removal of the second eye (Table 3). As in series 2.2 KN increased more than SN on the first day and this difference was greater and more persistent than in series 2.2, as would be expected on account of the higher absolute values. The ratio of SN/KN was back to normal on the sixth day.

TABLE 3

Concurrent estimates on six crabs of SN and KN after amputation of both eyes

Day:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
SN	43·08	44·02	37·31	39·51	39·83	33·53	27·44	30·91	28·01	20·62	19·17
KN	108·44	62·30	50·50	51·57	47·43	40·04	31·16	38·54	31·12	23·94	32·01
Ratio SN/KN	0·40	0·71	0·74	0·77	0·84	0·88	0·88	0·80	0·90	0·88	0·60

5.4. *Amputation of the eye-stalks by stages* (11 crabs, all fasting). Both eye-stalks were amputated in three stages: (1) at the level of the periphery of the corneal surfaces, (2) at the slight constriction, half-way down the stalk, and (3) at the basal articulation of the stalk. The first section leaves the endocrine complex intact, and the second removes the sinus gland, but not the proximal ganglionic part of the endocrine complex, located in the proximal half of the stalk.

Series 5.41: following the first section, the output was significantly above normal on the first day ($t=3{\cdot}19$, $n=10$), and fell significantly on the second ($t=3{\cdot}14$) to a value below normal (Text-fig. 8A). There was an increase on the third day as in a number of other series (Text-figs. 1D; 4 B, C; 5A; 9A), but this was not statistically significant. A phase of slower decline followed, which may correspond to phase 2 in other series. After the sixth day, apart from a small irregularity, output remained steady with a slight further decline. It was eventually significantly below normal. Student's *t*, in the test for this, increased from 2·08 on the sixth day, through 2·61 on the ninth to 3·58 on the twelfth. The overall trend was approximately exponential as in other series. In no other series was output so significantly subnormal through so much of the period, and it seems not improbable that the endocrine complex situated more proximally is normally antagonized, in this aspect of metabolism, by some agency in the intact tips of the eye-stalks.

Series 5.42: following the second section, in seven survivors of the first, the response contrasted remarkably with that to the first section, and was virtually identical with that of series 5.2 and 5.3, the initial increase in output being a little smaller and declining somewhat more rapidly to reach the normal value by the thirteenth day or so. In Text-fig. 7D these results are pooled with those of series 5.2 and 5.3.

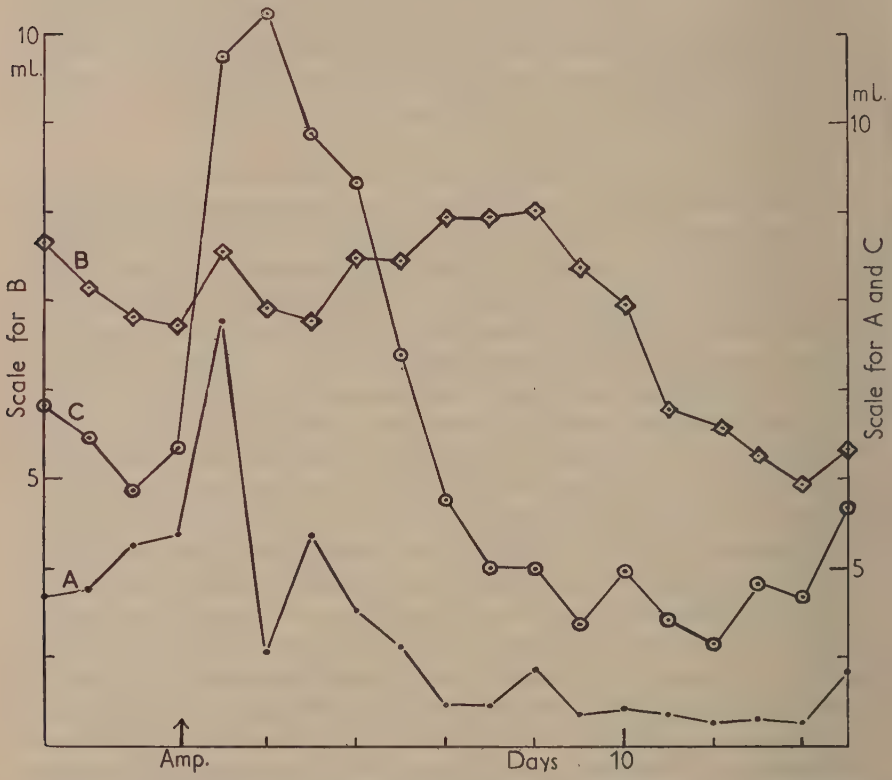
Series 5.43: only three of the seven individuals survived for 15 days after the third section. The initial increase in output was probably as great as after the second section, but the subsequent decline appeared to be more rapid, and output was almost normal by the fifth day, as in a typical case of leg-amputation. It seems probable that the organ normally depressing N-output is the sinus gland, and that following its removal the function is taken over by other organs, not located entirely in the proximal part of the eye-stalk. After the fifth day there was a period of slightly raised, if somewhat irregular, output, as in the other sections of series 5 (apart from 5.41). It probably represents phase 3 of other series. In the pooled results for series 5 the irregularities were smoothed out, and against the baseline of the overall exponential decline there was a phase of sustained, moderately raised, output between days 5 and 10.

6. *Second antennae: excision at the base* (17 crabs, all fasting)

Complete excision of the antennae damages the distal portion of the antennal gland, and a subsidiary aim of the present series was to ascertain whether or no the antennary gland is concerned in N-excretion (Prosser *et al.*, 1950, p. 197). Notwithstanding the extent of damage the increase in output of SN on the first day after operation (Text-fig. 5B) was less than in any previous series and was unique in not being statistically significant ($t=2{\cdot}63$, $n=16$). Moreover, on the second and subsequent days, output was subnormal and, in contrast to that in other series, except 5.41, remained so throughout the period, though recovering slowly. Records for the first eleven individuals indicated that output was significantly subnormal on the third day ($t=3{\cdot}55$, $n=10$), but the addition of the remaining six records reduced the value of t to $2{\cdot}75$, possibly owing to the increased damage of a deeper excision of the basal segment of the antennae. The very early, persisting subnormal output was unparalleled among isolated individuals of any other series, and the subsequent general upward trend was not found in any other series of isolated fasting individuals; it seems very probable, therefore, that some N is normally excreted via the antennal gland and that the duct of the gland is blocked temporarily by the operation. Later, output gradually increased, perhaps because the duct became patent again, or because the gills increased their contribution to N-excretion. N-retention itself does not necessarily increase subsequent output very much (Needham, 1955), but might account for the small increase observed here. The small peaks in output on days 5 and 10 conceivably may indicate phases 2 and 3 of the general response to trauma, superimposed on the specific effect of antennal duct-occlusion.

7. *Excision of portions of the body-wall*

7.1: *Margin of abdomen* (17 crabs, all fasting). The amount of tissue excised was approximately equal to that of two eye-stalks and the surface-area of the wound about equal to the sectional area of one walking-leg. The records show striking differences from that following limb-amputation. There was very little increase in mean SN-output on the first day (Text-fig. 8B), and output was again normal during the next 2 days. It then increased progressively to a higher maximum around the sixth to eighth days, though the increase was not statistically significant ($t = 1{\cdot}77$, $n = 16$). There followed a marked decline, and output on the eleventh day was significantly below that on the eighth ($t = 3{\cdot}92$, $n = 16$). It was not significantly below normal, however, even on the fourteenth day ($t = 2{\cdot}36$). Subdivision of the results into two subgroups gave two essentially similar graphs.



TEXT-FIG. 8. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). *A*, amputation of tips of both eye-stalks (11 crabs); *B*, excision of portion of abdominal margin (17 crabs); *C*, excision of portion of carapace margin (8 crabs).

7.2. *Margin of carapace* (8 crabs, all fasting). The amount of tissue removed was equal to that in the previous series but the area of the wound was approximately twice as great because the body-cavity is much shallower in the abdomen. Haemorrhage was considerably greater in this series. The initial increase in mean output (Text-fig. 8C) was approximately equal to that of series 3. It was not maximal until the second day, a feature paralleled only in the fasting section of series 4 (Text-fig. 6B). This was probably not fortuitous in the present series since it occurred in six of the eight crabs. The subsequent decline in output was steep and reached normal on the sixth day. Subsequently output was subnormal, but not significantly so ($t = 2{\cdot}42$ for the difference on the ninth day). There was no clear indication of a second cycle of raised output, and the record differs strikingly from that of other series. The single brief cycle of very high output may correspond to both cycles of series 7.1, telescoped into a much shorter time-scale. This telescoping might account for the position of the maximum, on day 2 rather than day 1, and for the earlier onset of the final increase than in 7.1.

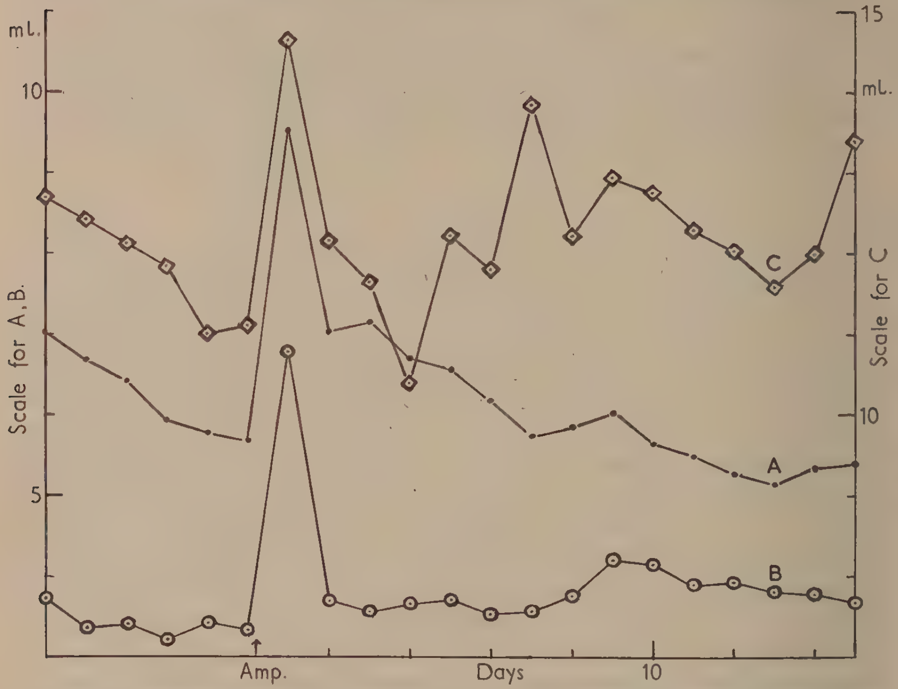
DISCUSSION

The response to wounding in this animal typically involves a considerable increase in N-output on the first day followed by a fluctuating return to normal over a varying period, which was not usually greater than 15 days, except after severe injury (series 3.1) and after certain special operations (series 5). Occasionally output was not maximal until the second day. The subsequent decline in output in an isolated fasting individual conformed very approximately to an exponential type of curve (Text-fig. 9A). Among grouped fasting crabs output returned rapidly to normal (series 1) or below (series 3.1), and subsequently followed an overall upward trend. This was probably due to mutual depression of output by the crabs, since depression by occlusion of the duct of the antennal gland (series 6) resulted in a generally similar record (Text-fig. 5B). In protein-fed crabs output returned relatively quickly to normal, or even to below normal, by the fourth day (Text-fig. 3B), but subsequently followed a general progressive increase, and was above normal at the end of the period. Protein food, therefore, appears to depress output in the early stages and to increase it later. This implies the existence of two distinct, and probably even contrasting, phases of metabolism (cf. p. 193). Bread appears to depress output throughout (Text-fig. 2), but more in the second than in the first half of the period.

Increased damage to tissues remaining at the site of amputation caused a larger initial increase in N-output, and a slower return to normal (Text-figs. 4 and 5A). Consequently, in series 3.2 the general trend approximated more to a linear than to an exponential decline. By contrast, spontaneous autotomy, involving minimal damage to remaining tissues, caused virtually no increase, in fasting animals, and no evident trend subsequently.

Amputation of eyes and antennae evoked responses peculiar to those organs, though superimposed on this the general response to wounding was probably detectable. The response to excision of parts of the body-wall differed from that to amputation of appendages in a later development of maximal output, on the second day in series 7.2 and the sixth to eighth days in series 7.1, in a persisting low output in the later part of the period studied, and in further details.

Taking as an index of the overall effect on N-output the average daily excess in output over normal, during the first ten days after operation (Table 4), the value for the carapace-wound was about equal to that for amputation of a pair of walking-legs (series 1 and 2), where the area of the wound was approximately the same. For the abdominal wound (series 7.1), of about half the area, the value was approximately half of this. It therefore seems probable that area of wound, or the extent of damage, which is proportional to this, is a major factor determining the excess output. By contrast, the amount to be regenerated is irrelevant, since this was much smaller for the body-wall operations than for the limb-amputations. Again the excess after amputation through the propus (series 3.2) was nearly twice that after amputation at the autotomy-plane (series 1, 2); in the



TEXT-FIG. 9. Mean daily output per individual crab of NH_3, expressed in ml. of N/100 solution, during the last few days before, and the 15 days following, amputation (*Amp.*). Pooled results: *A*, isolated fasting individuals (166 crabs); *B*, grouped fasting individuals (137 crabs); *C*, isolated protein-fed individuals (37 crabs).

TABLE 4

Mean excess in N-output over normal per day during first ten days after trauma in Carcinides

Series	1	2.1	2.2	3.1	3.2	4	5.1	5.41	5.2	5.3	6	7.1	7.2
% Excess	13	15	16	41	27	3·6	21	−20	128		−3·5	11	16

former the damage was greater but the amount to be regenerated much less. Amputation proximally to the autotomy-plane (series 3.1) caused a greater excess than that through the propus where the limb is narrower, and the wound therefore smaller. Spontaneous autotomy (series 4) caused only 3·6 per cent. excess (average for both fasting and feeding animals).

The excess output was little affected by differences in diet (series 1, 2.1, 2.2). However, the normal output was relatively high in protein-fed and low in carbohydrate-fed crabs, so that the absolute excess varied similarly. Excess therefore tended to a constant proportion of normal output: protein and carbohydrate food affected the emergency metabolism to the same extent as normal metabolism.

Removal of one eye caused less damage than amputation of two legs or than excision of parts of the body-wall, yet the average excess N-output was 21 per cent., and therefore there was a slight specific effect of removing the one endocrine complex. Removal of the second eye-stalk resulted in an excess of a higher order of magnitude. Removal of only the tips of the eye (5.41) caused a 20 per cent. deficit in output over the 10 days, so that there is little doubt of a specific effect on N-output, antagonistic to that of sinus-gland removal (p. 202). Amputation of both antennae resulted in a small deficit, also, which confirms the earlier conclusion that this operation specifically depresses N-output.

For traumatized mammals excess N-outputs from 30 per cent. (Cuthbertson, 1946) to 43 per cent. (Crofts & Peters, 1945) have been recorded, comparable to the values for the more extensive degrees of damage in the present experiments. In mammals the excess is too great to be due solely to N from demolished tissues, and this is probably true also for *Carcinides*. The maximal weight of tissue damaged could scarcely be more than 50 mg. per leg, i.e. the equivalent of 11 ml. of N/100 NH_3 (N = 16 per cent. of protein, which is 10 per cent. of the total weight). The actual excess for 10 days was 20·3 ml. in series 3.1, and 21 ml. in 3.2, and even then output was not back to normal, so that about half of the excess was due to other causes. In series 4, with minimal damage, the total excess for 10 days was only 3·3 ml. so that both components of the excess must increase with the degree of damage. This would support the idea of a wound-induced 'traumatic fever' (Cuthbertson, 1946).

In detail the relationship of the changes in N-output in this animal to those in mammals is not entirely clear. From its general time relationships the excess output during the period studied could correspond to N-flow in mammals, but in the latter there is also an initial period of depressed output (the 'ebb' period) lasting up to 3 days. In some individuals of *Carcinides*, output was not maximal until the second day, but in general an ebb period, if it exists, must be shorter than 1 day, or alternatively it is masked by the initial phase of high output, which does not affect mammals. Separate estimates of N-output for the first and second halves of the first day, showed a somewhat higher value for the second half, whereas in a normal animal the reverse is true (Needham, 1955), so that there may be a short ebb period.

The ebb and flow periods in mammals have been compared (Cuthbertson, 1946) with the phenomena of shock and countershock, which follows stress of any kind (Selye, 1948, 1950). Shock is characterized by a generally depressed metabolism including N-output, and countershock by the contrasted syndrome. In *Carcinides* there was some slight independent evidence of shock, in a reduced appetite, but the condition may be much less severe than in mammals. One useful function of shock is to reduce haemorrhage and the Crustacea, having a low blood-pressure and, on the other hand, good provision for sealing wounds, may have less 'need' for this response.

The possibility remains that the apparent absence of an ebb period and the high peak in output on the first day may be due to haemorrhage, which would not contaminate the excreta of mammals. The amount of haemorrhage in *Carcinides* was certainly sufficient to mask an ebb effect on the first day, while the slight trough on the second day and the subsequent increase in many of the records would be the appropriate indication of the existence of such an effect on the previous day. At the same time there was evidence that haemorrhage did not account for more than a fraction of the high excess output on the first day. Output was as high in the second half of the first 24 hours (p. 207), when haemorrhage had ceased, as in the first half, even if the water was not renewed (see Needham, 1955) between the two periods. In the fasting members of series 4, in a number of individuals of other series, and in series 7.2, where haemorrhage was at least as serious as in any other series, output was not maximal until the second day. Injuries without open wound also evoke a high excess on the first day (Needham, 1955), and a generally similar record subsequently. In passing it may be noted that eye-stalk amputation changes the response quantitatively rather than qualitatively, and so may affect the same mechanism, therefore a positive response-mechanism, as trauma in general.

It is possible to calculate the contribution of haemorrhage to the first day's output, knowing the values of SN and KN and the ratio SN/KN for blood and normal excretory water. The SN-content of 1 ml. of shed blood was found to be equivalent to 2·8 ml. of N/100 NH_3, a value in agreement with that of Delaunay (1931, p. 286) for non-protein N of blood, and increased very little (0·5 ml.) on standing 24 hours in sea-water. Assuming 0·8 g. per cent. as the value of total N of blood, then SN/KN is approximately 5 per cent., compared with 86 per cent. (Needham, 1955) for excreta. In series 2.2 (Table 2) the values of SN and KN for day 1 were 15·07 and 28·98 ml., so that if x, y ml. be the contributions of blood and excreta respectively to KN, then

$$\begin{aligned} 0{\cdot}05x + 0{\cdot}86y &= 15{\cdot}07 \\ x + \qquad y &= 28{\cdot}98, \end{aligned}$$

giving $x = 12{\cdot}1$ and $y = 16{\cdot}9$. The contributions of blood and excreta to SN, therefore, were 0·6 and 14·5 ml. respectively, blood therefore accounting for 12 per cent. of the *excess* SN. The value of x indicates 0·2 ml. as the actual volume of blood shed. The same calculation applied to the output on the first day after puncture with a hypodermic needle, of approximately the same bore as the limb-artery piercing the autotomy membrane, indicated a loss of 0·13 ml. of blood. For series 5.2, $x=61{\cdot}9$ and $y=46{\cdot}5$; blood again contributed only 12 per cent. of the excess SN, but the total haemorrhage was 1·1 ml.

There seems little doubt, therefore, that the high output on the first day was not due solely to haemorrhage. It may depend on the type of wound rather than on taxonomic differences since the response of series 7, in particular of 7.1, resembled the mammalian response which also has been studied mainly on wounds of body-wall and of bone. In such wounds the main difference from limb-amputation, of possible significance, is the smaller number of nerve fibres sectioned.

TABLE 5

Time-tables, in days from the time of operation, of the basic events in the response of N-output to various forms of trauma, in Carcinides. *A statistically significant change in output is indicated by S*

Series	*Peak of phase 1*	*Trough between phases 1 and 2*		*Peak of phase 2*		*Trough between phases 2 and 3*		*Peak of phase 3*		*Trough between phases 3 and 4*	
1	1	2		? 2	S	7	S	10		12	
2.1	1	?		?	S	4–6		9		13	
2.2	1	?		?		4	S	7	S	13	S
3.1	1	2		3 or 5	S	6		10		14–15	
3.2	1	2		3	S	4	S	5		13	
4	1	3		5	S	6		9		13	
5.1	1	2		3		5		9		13	
6	1	3		5		6		10		?	

It seems clear that there were further significant details in the present records, and that in order to describe and compare effectively the responses of the various series it is necessary to recognize, however modified by particular treatments, a basic pattern of four phases of raised output (p. 193). There seems little question of the demarcation between the first phase, of steep increase and decrease, and the second phase of slower decline towards normal output, often with a moderate increase initially. In series 4, where damage was minimal, an increase, between days 3 and 5, was statistically significant at the 2 per cent. level. The decline towards the end of phase 2 was usually significant, and the final increase (phase 4) also was very consistent in the various series, but it is less certain that a cycle of raised output between these two phases was a significant and general feature. In some series (Text-figs. 1D; 8 A, C) it was certainly slight or absent, and in other series it was consistent only with some variability in time-scale, but these may be partly sampling errors and partly significant differences in response to the different treatments. Comparable comparative studies on other animals would be valuable. A complete typical pattern of four periods seemed evident in series 1, 2.1, 3.1, 5.1, and 6, and, with greater differences in time-scale, in series 2.2, 3.2, and 4. A cycle of relatively higher output, with the time-relations of phase 3, was detected even in the pooled results of series 5. The response in series 7 did not fit this pattern in detail, but those of other series did so with considerable certainty and synchronization (Table 5). In Table 5 uncertainty is indicated by (?) and by wide time-bands; on the other hand, the statistical significance of particular transitions between phases has been indicated by 'S' (except the transitions to and from phase 1, which were usually too obviously significant to require tests). Time-scales naturally become increasingly asynchronous with time from the date of operation, both between and within series.

In view of the variations in time-scale and other features, due to deliberately diverse experiments, the pooled results of all series could not be expected to give a very clear basic pattern of response, but, nevertheless, those for fasting isolated individuals (Text-fig. 9A) did give an indication of these four phases, and the period of phases 2 and 3 was consistent with that given by the correlogram (p. 193). In this figure phase 4 was seen to be the beginning of a return to normal from a period of generally subnormal output. In the pooled results for grouped, fasting animals (Text-fig. 9B) there was a fairly clear indication of phases 2 and 3, superimposed on a general trend which differed from that of isolated crabs (p. 193).

The pooled results for isolated protein-fed animals (Text-fig. 9C) differed from those for series 2.2 in the presence of an inflexion on day 2 and in that the increase in mean output between days 6 and 7 was significant ($t = 2{\cdot}88$, $n = 36$), thus strengthening the view (p. 196) that the record between days 2 and 4 corresponds to phase 2 of other series and that there is a single phase of increase subsequently, between days 4 and 7, so that the whole period between days 4 and 13 corresponds to phase 3 of other series. The deep trough on day 4 may have been due largely to a tendency to lose appetite around the second day, output lagging on intake-variations (Needham, 1955). The record also indicated that protein probably increases the amplitude of all output-responses. Carbohydrate-food, on the other hand, tends to depress output at all times.

Other workers have observed similar fluctuations in N-output, and in other relevant processes, during the corresponding period of regeneration and wound-healing in other animals. Cuthbertson *et al.* (1939) found two cycles of raised N-output during flow, in mammals, with their peaks on the fourth to fifth and eighth days. Orechovitch (1936) found two peaks in dipeptidase activity in the tissues of the regenerating tail of *Pelobates*, on the second and tenth days: corresponding peaks in N-output might be anticipated. Cuthbertson *et al.* (1939) obtained similar and even more complex fluctuations in some metabolic processes, for instance in creatine-output. The blood-sugar of rats, subjected to the stress of low external temperature (Selye, 1946), rose and fell on the first day, rose again on the second and declined progressively to the sixth day, and then rose to a third peak on the tenth day before a final decline to normal—a remarkably close resemblance to the pattern of N-output in the present results. It is at least probable that N-metabolism shows fluctuations corresponding to those of carbohydrate-metabolism. In *Carcinides* this type of pattern is not peculiar to the response to trauma, but is a general property of the response of N-output to any change in environmental conditions (Needham, 1955). The details of the pattern may be peculiar to specific conditions, however, as the present results indicate.

An increase in output after removing the eye-stalks is consistent with the observation of Nieland & Scheer (1953) that the amount of protein in the body decreases under these conditions. A variation in blood-protein during the moult-cycle (Leone, 1953) no doubt depends on the activity of the eye-stalk endocrines, since one of their main functions is the control of this cycle. The present experiments indicate (p. 207) that some factor present in the tips of the eyes normally antagonizes the sinus gland complex.

SUMMARY

Amputation of appendages in fasting *Carcinides* causes an immediate large increase in nitrogen output which then declines along an exponential type of curve, during the next 12 days or so. Superimposed on this curve are cyclic fluctuations in output, with a period of about 6 days. The excess output over the first 10 days is proportional to the normal output before operation, the relative excess being independent of diet. Later, however, carbohydrate reduces output and protein increases the short-term oscillations in output. Excess nitrogen output is related to the amount of damaged tissue remaining but not to the amount of tissue removed. It is not due entirely to the nitrogen from demolished tissues. Removal of the sinus gland region of the eye-stalks causes an increase in output greater by one order of magnitude than that due to limb-amputation. By contrast, amputation of the tips of the eyes only depresses output. Extirpation of the second antennae depressed output slightly, probably indicating that the antennal gland normally excretes nitrogen. After excision of portions of the body-wall the pattern of output differs from that following amputation of appendages, and is more similar than the latter to that following skin and bone trauma in mammals.

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REFERENCES

ANON. (1953). Methionine and cystine in wound-healing in albino rats. *Nutrition Rev.* **11**, 124–6.

CARLISLE, D. B. (1954). *Comparative Studies of the Pituitary and Pituitary-like Organs in Chordates and Crustaceans.* Thesis presented for the degree of Doctor of Philosophy, University of Oxford.

CROFTS, P. B., & PETERS, R. A. (1945). Nitrogen-loss after thermal burns: effects of adding protein and methionine to the diet of rats. *Lancet*, **288**, 266–72.

CUTHBERTSON, D. P. (1942). Post-shock metabolic response. *Lancet*, **242**, 433–6.

—— (1946). Dietary protein in relation to convalescence from injury. *Proc. Nutr. Soc.* **4**, 185–93.

CUTHBERTSON, D. P., McGIRR, J. L., & ROBERTSON, J. S. M. (1939). The effect of fracture of bone on the metabolism of the rat. *Quart. J. exp. Physiol.* **29,** 13–25.

DELAUNAY, H. (1931). L'Excrétion azoté des Invertébrés. *Biol. Rev.*, **6,** 265–301.

KENDALL, M. G. (1951). *The Advanced Theory of Statistics.* Vol. 2 (3rd edit.). London: Charles Griffin.

LEONE, C. A. (1953). Preliminary observations on intraspecific variations in the levels of total protein in the sera of some Decapod Crustacea. *Science,* **118,** 295–6.

NEEDHAM, A. E. (1952). *Regeneration and Wound-healing.* London: Methuen & Co.

—— (1955). Factors affecting nitrogen-excretion in *Carcinides maenas. Physiol. comp. et Oecol.* (in the press).

NIELAND, K. A., & SCHEER, B. T. (1953). The influence of fasting and of sinus-gland removal on body-composition of *Hemigrapsus nudus. Physiol. comp. et Oecol.* **4,** 321–6.

ORECHOVITCH, W. N. (1936). Die Veränderung der Aktivität der Dipeptidasen in den regenerierenden Geweben von Amphibien. *Biochem. Z.* **286,** 91–92.

PROSSER, C. L., *et al.* (1950). *Comparative Animal Physiology.* Philadelphia: W. B. Saunders Co.

SELYE, H. (1946). The general adaptation syndrome and the diseases of adaptation. *J. clin. Endocrinol.* **6,** 117–230.

—— (1948). *Textbook of Endocrinology.* Montreal: Acta Endocrinologia.

—— (1950). *Stress.* Montreal: Acta Inc.

SHOLL, D. A. (1954). Regularities in growth-curves, including rhythms and allometry. In *Dynamics of Growth Processes.* Ed. E. J. Boell. London: Oxford University Press.

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The Development of the Otoliths of the Mouse

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WITH TWO PLATES

INTRODUCTION

THERE is still little known about the formation of the mammalian otolith. Work carried out on this problem many years ago (Nishio, 1926) left many questions unanswered. In the meantime considerable knowledge has been gained of the development of other calcified structures, namely, bones and teeth, and the present paper consists of a reinvestigation of the development of the otoliths of the house mouse (*Mus musculus* L.) in the light of this new knowledge of calcification.

The normal fully formed otoliths of the mouse consist of a flat mass of small prismatic calcareous crystals in an organic matrix. The calcium salts of the otoliths of all vertebrates are present as calcium carbonate in the form of aragonite. Hastings (1935), working with the salamander, *Amblystoma tigrinum*, found in addition 15·9 per cent. of calcium phosphate in an apatite form. The organic matrix is said to consist of protein. It forms an intimate part of each crystal and fills the spaces between them. According to Tenaglia (1925) it is of a semi-solid consistency and imparts a definite shape to the otolith. Embedded in it are the tips of the sensory hairs of the macula which the otolith overlies. An otolith is present in the sacculus and in the utriculus of each ear.

Questions for consideration in a study of the development of the otoliths include the following: which part of the wall of the labyrinth secretes the matrix, what is the mechanism of deposition of calcium salts on the matrix, and why are they deposited only on the matrix? There might be a local secretion of calcium salts by the labyrinth wall, a local absorption of water by the wall, or the matrix itself might actively take part in calcification.

Nishio (1926) studied the formation of the otoliths in various vertebrate groups and reviewed earlier work. Previous authors had generally reported droplets emanating from the surface of the macula and had considered these to be otolith material. Nishio, however, interpreted such droplets as fixation artifacts, and

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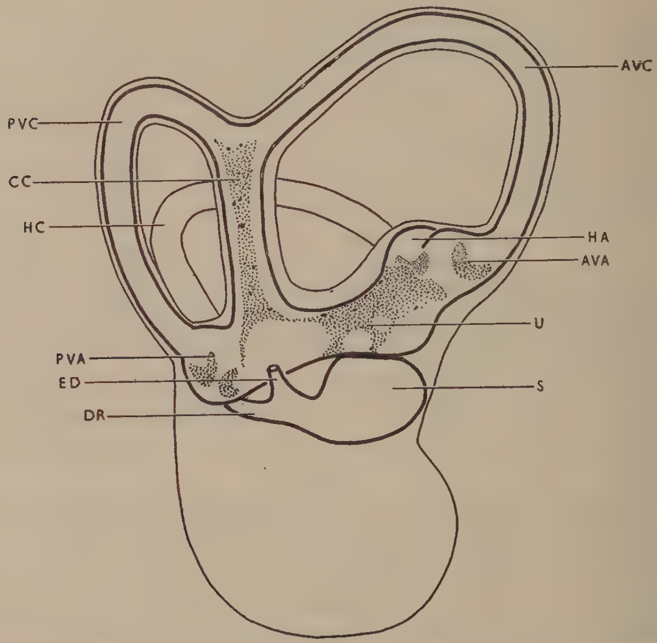
himself could not discover which parts of the labyrinth wall secreted the otolith material, though he suggested that the 'secretory regions' of the utriculus might play some part. These 'secretory regions' are certain highly vascular, pigmented regions (Text-fig. 1) of the utriculus, ampullae, and crus commune of the vertical canals which were described by Iwata (1924; and later Hazama, 1929), and to which a secretory function is attributed on histological grounds, although it is not known what substances are secreted. In the mouse Nishio found that calcium salts and matrix appeared suddenly in foetuses of 17–18 mm. length, the matrix forming part of each crystal. In an attempt to demonstrate local enrichment of the endolymph with calcium he fixed foetal mice by injection with a mixture of sodium oxalate and formalin. He found masses of calcium oxalate crystals lying between the otolith and the surface of the macula and interpreted this as evidence of local enrichment with calcium ions. This method of detecting calcium in solution is open to two serious objections, however. First, the solute could be significantly displaced by diffusion before the oxalate was precipitated, so that the



Text-fig. 1. Distribution of the pigment of the secretory regions in the labyrinth of the mouse. Camera lucida drawing, seen from the medial side. ×25. *AVA*, anterior vertical ampulla; *AVC*, anterior vertical canal; *CC*, crus commune; *DR*, ductus reuniens (to cochlear duct); *ED*, endolymphatic duct (to endolymphatic sac); *HA*, horizontal ampulla; *HC*, horizontal canal; *PVA*, posterior vertical ampulla; *PVC*, posterior vertical canal; *S*, sacculus; *U*, utriculus. The cochlear duct is not shown.

localization would be inaccurate. Secondly, Nishio's belief that no calcium phosphate or carbonate would be dissolved and reprecipitated as oxalate by the oxalate-formalin mixture is probably unjustified. These objections are unfortunately sufficiently serious to vitiate Nishio's conclusions. Hence, neither the mechanism of secretion of the matrix nor of deposition of calcium salts can be considered established by Nishio's work.

The present work on the development of the normal mouse otolith was undertaken in conjunction with a study of the development of hereditary absence of otoliths in mice (Lyon, 1951, 1953). The development of the ears of the mutant mice will be considered in a later paper. Since Nishio's work it has been learned that in the formation and calcification of bone the presence of phosphatase, of the polysaccharide bone matrix, and of glycogen are all essential, as well as of a suitable concentration of inorganic salts (Dallemagne, 1950). Staining techniques for phosphatase and polysaccharide were therefore used in the present work, as well as birefringence methods intended to detect the earliest appearance of calcium salts.

In addition, the cupulae and the tectorial membrane, the covering membranes of the cristae and organ of Corti respectively, were studied, since these membranes bear the same relationship to the sensory areas to which they belong as the otolith does to the macula.

MATERIALS AND METHODS

Timing embryos, dissection, fixation, and microtomy

Mouse embryos of 14½–18½ days' gestation and young mice from birth to 4 days old were studied. They came from a stock segregating for the recessive mutant *pallid*, which when homozygous produces absence of otoliths. All the animals considered in this paper, however, were heterozygotes and therefore had normal ears. (Homozygous embryos of more than 11½ days' gestation can be recognized by the absence of pigment from their eyes.)

The stage of gestation of foetuses was determined by daily inspection of mated females for vaginal plugs and was checked by the external features (Grüneberg, 1943). Post-natal stages were timed from birth, inspection for births being made once daily.

To obtain foetuses, pregnant females were killed with ether and the young were removed and separated from their membranes in Ringer's solution. They were then decapitated and only the heads were fixed. For stages after birth the animals were anaesthetized or killed with ether and decapitated. The ears and surrounding parts of the skull were dissected out and fixed by immersion.

Bouin's fluid, Susa, 80 per cent. alcohol, and 4 per cent. formaldehyde in 0·9 per cent. sodium chloride neutralized with sodium hydroxide were used as fixatives. Specimens fixed in Bouin or Susa were decalcified by the fixative.

The other specimens were not decalcified since at these early stages paraffin-embedded material can easily be sectioned without decalcification. All specimens were embedded in paraffin and sectioned transversely at 7μ; some of the post-natal material was previously infiltrated with 1 per cent. celloidin in methyl benzoate, to give additional support to the tissues.

Staining and histochemical methods

(*a*) *Alkaline phosphatase.* Gomori's (1939) staining method for alkaline phosphatase was used on specimens fixed in ice-cold 80 per cent. alcohol. The sections were incubated with sodium glycerophosphate for 6 hours and the calcium phosphate precipitated by the enzyme was made visible by substitution with cobalt sulphide. Preformed calcium phosphate, present in the bones of the skull, had first to be removed; this was done by taking the sections down to water, decalcifying in Lorch's (1947) solution for 15 minutes and washing thoroughly.

It was then necessary to ensure that the decalcification had been complete and that subjection to the low pH of the decalcifying solution had not inactivated the enzyme. Slides treated with Lorch's solution and then incubated in a control medium served to ensure the completeness of decalcification. Confirmation that prior decalcification did not reduce the phosphatase activity was obtained by comparing undecalcified slides incubated in substrate medium with similar slides incubated in control medium and decalcified slides incubated in substrate medium. No appreciable inhibition of phosphatase activity was detected.

(*b*) *Polysaccharides.* The staining method used to demonstrate polysaccharides was the McManus (1948) variant of the Hotchkiss-McManus ('PAS') method, for the oxidation of 1–2 glycol groups with periodic acid, and staining of the aldehydes formed with Schiff's reagent. Specimens fixed in formalin, alcohol, Bouin, and Susa were stained by this method. Delafield's haematoxylin and light green both proved suitable as counterstains.

Control slides were placed in distilled water instead of periodic acid. Examination of these confirmed that the sections contained no substances which could recolourize Schiff's reagent without previous oxidation. In order to determine what part of the response to PAS could be attributed to glycogen, some slides were incubated at 40° C. for 15 minutes with a strong solution of human saliva, and then rinsed, before being placed in the periodic acid. Those PAS-reacting substances which were removed by the salivary amylase were considered to be glycogens.

(*c*) *Calcium salts.* Staining the inorganic part of the developing otoliths proved difficult and various methods were tried.

Some stains which gave good staining of the calcium of the developing bone present in the sections failed to stain the inorganic part of the otoliths. Such stains included alizarin red S, gallamine blue (Stock, 1949), and cobalt sulphide. Recourse was therefore made to the less specific and less sensitive stains, Delafield's haematoxylin and the von Kossa calcium stain. Both these gave a reaction, and the reactions could be prevented by previous decalcification. (In the case of haematoxylin this is in contrast to the findings of Cameron (1930) who stated that the staining of calcified tissues with haematoxylin was not prevented by decalcification. In the present work calcium deposits in bone and otoliths were stained a deep royal blue by haematoxylin, a colour easily distinguished from the light blue which might be seen after decalcification.) Alcohol- and formalin-fixed material was used for these tests.

For a more sensitive detection of calcium salts some slides of alcohol-fixed sections were taken from xylol to absolute alcohol, to eosin in 95 per cent. alcohol, then returned via absolute alcohol to xylol and mounted. This process was intended to minimize the loss of salts by solution during staining. The calcareous matter could then be seen, unstained, either by normal light as highly refractile crystals or, with the polarizing microscope, as birefringent particles.

(*d*) *Ear morphology.* Delafield's haematoxylin and eosin (H & E), Masson's haematoxylin-ponceau-light green trichrome stain, and Mallory's connective tissue stain were all used in studying the general morphology of the ear.

RESULTS

The first and main section will describe the development of the otoliths as seen after the various types of fixation. The development and staining reactions of the walls of the sacculus and utriculus will also be described, since changes in the walls may throw light on the origin of the otolithic material. A final section will deal briefly with the appearance of the cupulae and tectorial membrane. Table 1 shows the material available.

TABLE 1

The number of specimens, fixed and stained in various ways, which were available at each stage of development

Fixative	*Stain*	*Stage in days*								
		14½	15½	16½	17½	18½	*Birth*	1	2	3
Alcohol	Phosphatase	—	6	—	5	—	1	—	—	1
	PAS	8	4	3	2	—	—	—	—	1
	Birefringence	3	6	4	3	—	—	—	—	—
	H and E	1	1	1	—	—	2	—	—	—
Formalin	PAS	1	3	4	—	1	—	—	—	—
	H and E	3	8	1	5	2	—	—	—	—
	Masson or Mallory	2	4	7	—	—	—	—	—	—
	Kossa	—	2	4	1	—	—	—	—	1
Bouin or Susa	PAS	1	2	1	1	—	—	—	—	1
	H and E	—	—	1	1	—	15	5	5	2
	Masson or Mallory	2	—	—	2	—	2	8	8	10

Observations on the developing otoliths will be described by stages beginning with the earliest available, i.e. 14½ days' gestation. At this stage all the main parts of the inner ear had been formed and the sensory epithelia of the otolith organs, ampullae, and cochlea had been differentiated from the surrounding wall, though not of course fully differentiated. The secretory regions of the utriculus and canals were not yet pigmented but already had a rich vascular supply. The lateral wall of the sacculus, on the other hand, had a poor blood supply.

Of the covering membranes of the sensory epithelia the cupulae were already formed, and the tectorial membrane was also present. In alcohol-fixed specimens there was also something representing the developing otolith in the lumen of the sacculus and utriculus. In formalin- and Bouin- or Susa-fixed specimens nothing was yet visible, making it clear that these fixatives did not preserve this part of the developing otolith. In fact, alcohol fixation showed the otolith to consist of at least three parts: a precipitate, probably fluid or gelatinous in life; granules of calcium salts; and an organic matrix forming part of each calcium granule. Neither formalin nor the acid mixtures preserved the gelatinous layer and both of these at various stages of development also failed to preserve other parts of the developing otolith. Thus, alcohol fixation seemed to give the fullest picture of otolith development and therefore in the following account alcohol-fixed specimens will be described in most detail and animals fixed with other solutions will be compared with alcohol-fixed ones.

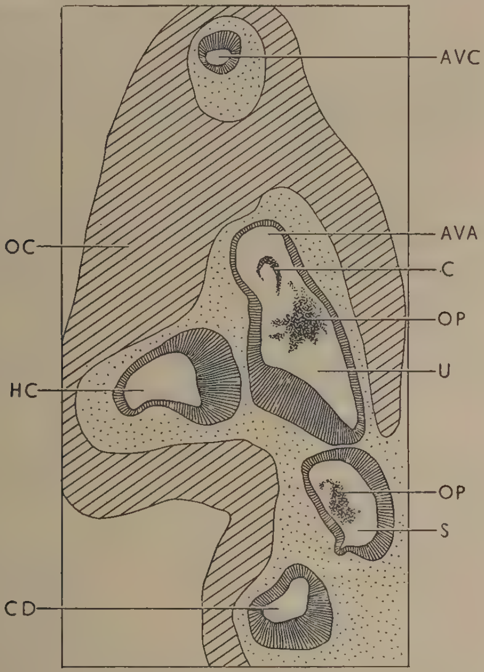
Development of the otoliths

(*a*) *14½ days' gestation.* At this stage, in alcohol-fixed specimens, there was present in the lumen of both the sacculus and the utriculus something appearing to be a fixation precipitate of a protein (Text-fig. 2). That is, the bulk of this substance presented no very definite form, either filamentous or granular, but merely looked like a loose network. In the midst of it, however, a few small pieces of membranous material were visible, particularly in the utriculus.

When stained with Delafield's haematoxylin and eosin the whole of this pre-otolithic material took up the pink eosin stain. With PAS, however, the protein precipitate was negative and took up only the counterstain, light green, whereas the membranous specks were PAS-positive and stained a pink to mauve colour. In addition, strongly PAS-positive droplets were scattered all over the protein precipitate, and were also found to a small extent free in the lumen of the sacculus (Plate 1, fig. C). These droplets could be removed by salivary amylase and were therefore considered to be glycogen.

The animals of one litter were slightly more advanced than the rest and had more PAS-positive membranous material. In addition, two of them had a few small PAS-positive granules in the utricular precipitate, lying along a PAS-positive membrane. These were considered to be the first sign of the developing otolith granules.

Birefringence was tested in eosin-stained slides of one animal from one of the younger litters and two from the older litter. In the younger animal no birefringence was found either in the lumen or walls of the sacculus or utriculus. In both the older ones the utricular precipitate showed birefringence in the form of a cloud of minute pin-points of weak birefringence lying in the precipitate itself. Each pin-point was much smaller than an otolith granule and nothing resembling otolith granules was seen. Slides which had been through the PAS routine, and hence had been treated with acid solutions, showed no birefringence.



TEXT-FIG. 2. Transverse section through the membranous labyrinth of a 14½-day mouse embryo. Camera lucida drawing. ×85. *AVA*, anterior vertical ampulla; *AVC*, anterior vertical canal; *C*, cupula; *CD*, cochlear duct; *HC*, horizontal canal; *OC*, otic capsule; *OP*, otolith precipitate; *S*, sacculus; *U*, utriculus.

The position of the pre-otolithic material in these preparations was very different from that of the fully differentiated otolith. In the sacculus, as Plate 1, fig. C shows, the mass lay near the lateral wall, opposite the macula over which the otolith finally lies, and wisps of material appeared to be in contact with the wall. In the utriculus, where the final position of the otolith is ventral, the pre-otolithic mass lay dorsolaterally, near the mouths of the ampullae of the anterior vertical and horizontal canal, and some of it was actually inside the ampullae rather than the utriculus. Again there were wisps in contact with the dorsolateral wall of the utriculus, and in fact some material appeared flattened against the wall.

In formalin-fixed specimens none of the developing otolith was preserved, and in three Bouin-fixed animals there were merely small pieces of PAS-positive membranous material in each sacculus and utriculus. In the sacculus they lay near the lateral wall, and in the utriculus near the macula. When stained with Masson's trichrome and with Mallory's stain they took up the light green and aniline blue respectively.

In alcohol- and formalin-fixed specimens all the cells of the lateral and dorsal walls of the sacculus, up to the edges of the macula, were rich in glycogen, in the superficial parts of the cells (Plate 1, fig. C). Some of it appeared to be escaping into the lumen of the sacculus, but this may have been a fixation artefact. The connective tissue applied to the lateral wall was also rich in glycogen. The utricular wall, on the other hand, contained only a little glycogen in the dorsolateral region.

Only five specimens showed any pigmentation of the secretory regions. It took the form of a few separate melanoblasts in the connective tissue of the dorsal and ventro-lateral utricular walls, a long way from the final position of the pigment layer.

(*b*) *15½ days' gestation.* The general appearance of the developing otoliths of alcohol-fixed animals resembled that at 14½ days (Plate 1, fig. D). Diffuse masses resembling precipitated protein still floated freely in the lumina of the sacculus and the utriculus. More glycogen was present, however, and also more PAS-positive amylase-resistant material. In some specimens this material was of no definite form, in others it looked membranous, and in some a few small granules were present, lying along a PAS-positive membrane.

Three eosin-stained specimens were examined with the polarizing microscope. In one, birefringence was absent from the inner ear. In the other two it was present in the pre-otolithic material, as at 14½ days, in the form of a cloud of pin-points, much smaller than otolith granules. The birefringence was stronger than at 14½ days, however, and the points were larger and much more numerous. In addition, birefringent material now lined the entire dorsal wall of the utriculus, the dorsolateral region of the saccular wall and the more dorsal part of the macula, and even the walls of the anterior and lateral ampullae and the basal coil of the cochlea.

Six animals were tested for the presence of alkaline phosphatase. Activity was present diffusely in the precipitate of both sacculus and utriculus (Plate 1, fig. A). In addition, the few granules present in the utriculus stained heavily. No attempt was made to assess accurately the strength of this phosphatase activity. In two specimens, however, a series of slides were incubated for varying lengths of time. Activity was detected in the perichondrium of the skull cartilages after 1 hour's incubation, and in calcifying cartilage, chorioid plexus, and brain tissue after 2 hours. It was not until after 5 hours' incubation that the pre-otolith showed cobalt sulphide staining. Thus, the activity in this material was not great.

The apparent position of the precipitated pre-otoliths had changed slightly by comparison with 14½-day specimens. Laterally, in the sacculus, and dorsally, in the utriculus, the material was still in contact with the walls. The mass was somewhat greater, however, and extended farther, medially or ventrally, towards the final position of the otolith.

In formalin-fixed specimens a little of the developing otolith was now preserved. There were a few filamentous specks (Plate 1, fig. B), which were PAS-positive, stained green with Masson's, an indeterminate colour with haematoxylin and eosin, and were negative to the von Kossa calcium stain. They were scattered through the lumina of the sacculus and utriculus. Only one specimen was Susa-fixed; it showed a few small PAS-positive specks lying near the maculae.

The lateral wall of the sacculus was still rich in glycogen at 15½ days, but in the utricular wall very little remained. Phosphatase activity was also present in the walls of the sacculus and utriculus, but in the maculae and not in the lateral or dorsal walls. The nuclei, cell surfaces, and hairs themselves of the hair cells of the maculae were all stained black by cobalt sulphide after incubation in substrate for 6 hours. Thus, as in the developing otolith, the activity was weak.

Pigment had now spread over the dorsal wall of the utriculus and a small area of the ventrolateral wall near the mouth of the ampulla of the horizontal canal, but the ramifications of the individual melanoblasts could still easily be made out. They were still not closely applied to the epithelial lining of the utriculus, but were scattered in the loose connective tissue.

(*c*) *16½ days' gestation.* The four alcohol-fixed animals studied were at rather different stages of development but in all a complete change in the appearance of the developing otolith had taken place since the 15½-day stage. There was now a more or less flat layer of protein precipitate surmounted by a layer of refractile calcareous granules of definite otolith shape. Each granule had an organic matrix, staining pink to mauve with PAS, and when the calcium salts were removed by acid treatment this matrix retained the shape of granules, though of a much smaller size. The protein or gelatinous layer had a somewhat more solid appearance than formerly; it was on the whole negative to PAS, but may have been positive at some points. One specimen was stained with haematoxylin and eosin; here the otolith granules stained heavily with haematoxylin, an indication of the presence of calcium salts, and the underlying gelatinous layer stained pink with eosin. In all specimens the calcium salts were highly birefringent (Plate 2, figs. I and J).

The edges of the developing otoliths still had the appearance that the whole had had at 15½ days. There was a diffuse PAS-negative precipitate intermingled with a PAS-positive amylase-resistant substance and with droplets of glycogen. (Glycogen was now absent from the regions where the calcareous granules had formed.) There was also a cloud of pin-point birefringence, in contrast to the granular form of the birefringent material in the more fully developed region.

The differences among the four alcohol-fixed specimens concerned chiefly the amount of calcareous material deposited and the general shape of the developing otolith. As the amount of calcium salts increased, from the apparently youngest to the apparently oldest specimen, the otolith changed in form from its original globular shape to the final flat lamellar form. In all four specimens the edges remained diffuse.

The apparent position of the developing otoliths had also changed greatly since 15½ days. The main bulk of each was now lying over and parallel to the macula to which it belonged, although neither otolith was yet as close to the macula as when fully developed. Although the main bulk of the otolith had moved, however, there were still wisps of material, including birefringent pin-points, near the walls, especially in the utriculus. There was much similar material, also birefringent, in the anterior or lateral ampullae, giving the appearance of having been displaced from the utriculus. In all four specimens, however, there were a few wisps and pin-points of birefringence which did not look displaced, lying in contact with the pigmented regions of the utricular walls, both dorsally and ventrally (Plate 1, fig. E; Plate 2, fig. I).

The formalin-fixed developing otolith also changed greatly in appearance between 15½ and 16½ days. At 16½ days there was a continuous layer of otolith matrix preserved in both the sacculus and the utriculus (Plate 1, fig. F). In contrast to the alcohol-fixed specimens, however, no calcium was detected either with haematoxylin or with the von Kossa stain. Another difference was that the organic material present was not separable into two layers, and although the layer present presumably corresponded to the granular matrix of the alcohol-fixed specimens it did not show the shape of the individual granules. The reaction to PAS was much stronger than in alcohol-fixed specimens; Masson's and Mallory's stains gave green and blue colours respectively. After formalin fixation the otoliths always lay near and parallel to the maculae.

Bouin still failed to preserve any part of the developing otolith.

In the walls of the sacculus and utriculus histological differentiation was leading to flattening of the cells of the lateral saccular and dorsal utricular walls, so that the glycogen of the lateral saccular wall was now less obviously superficial. Further development had occurred in the pigmentation of the utricular wall, and there was now a continuous layer of pigment, still not very closely applied to the wall. A new feature in the sacculus of the alcohol-fixed specimens was a PAS-positive membrane lining the lateral wall. Glycogen droplets were seen along it.

(*d*) *17½ days' gestation.* No radical change in the appearance of the alcohol-fixed otoliths took place between 16½ and 17½ days' gestation. More calcium salts were deposited and more organic matrix, so that after decalcification the matrix now retained the shape of full-size granules. The details of the shape of the fully developed otolith were beginning to be apparent; thus the thin central region of the fully developed utricular otolith could be detected in 17½-day foetuses (Plate 2, fig. G). At the edges of the otolith there was still the diffuse material seen at 16½ days, and wisps and birefringent specks were still seen in contact with the pigmented regions of the utricular wall.

The staining reactions to PAS of the gelatinous and granular components of the otolith matrix remained unchanged since 16½ days: the gelatinous layer was doubtfully negative and the granular matrix positive. Glycogen was no longer present in the matrix, though there was a little in the lumen of the sacculus near the lateral wall. After staining with Delafield's haematoxylin and eosin the gelatinous layer took up the eosin, and the calcium salts gave a dark blue stain. If the salts were removed by acid treatment, however, the granular matrix did not stain with either haematoxylin or eosin; it remained completely colourless and very difficult to see. When stained for alkaline phosphatase activity the granular matrix appeared uniformly greyish. The gelatinous layer, on the other hand, showed variable staining; it was in some places black, in others greyish, and in others again negative, having taken up only the eosin counterstain. In control slides the granular matrix remained completely unstained by the eosin counterstain and almost invisible. In undecalcified control slides the calcium salts also were unstained by the cobalt sulphide and were seen simply as refractile crystals.

The otolith granules were strongly birefringent in all specimens, and birefringence was absent from acid-treated slides. In one specimen, stained with alcoholic eosin without being taken down to water, pin-point birefringence was also still present in the material near the utricular wall. In two other specimens, the slides of which had been passed through water, such birefringence was missing, but it is not certain that birefringent material had not been dissolved by the watery solutions.

The position of both otoliths was now more or less the final one. Both were lying parallel and close to the maculae, although still not clearly in contact with the tips of the sensory hairs.

After formalin fixation the utricular otolith now gave a reaction for calcium with both haematoxylin (Plate 2, fig. H) and von Kossa, and when the calcium salts were removed by acid the matrix showed the granular form. In the sacculus the calcium salts were still not preserved and the matrix remained shapeless. However, when stained with haematoxylin and eosin it took on a light blue colour, in contrast to the complete failure to stain of the alcohol-fixed granular matrix.

Histological differentiation was continuing in the walls of the labyrinth. The cells of the lateral saccular wall had become flatter, but glycogen was still present. The cells of the dorsal and ventro-lateral regions of the utriculus had also become flatter, and a continuous layer of pigment was now closely applied to the wall. As in the 15½-day specimens the surface of the cells of the maculae of the sacculus and utriculus showed phosphatase activity. This activity was weak in both, and weaker in the sacculus than in the utriculus.

(*e*) *18½ days' gestation.* Only three formalin-fixed specimens were studied. These differed from 17½-day formalin animals in that calcium salts were now preserved in the sacculus as well as the utriculus.

(*f*) *Newborn.* In the three alcohol-fixed ears the chief change that had taken place in the otolith since the 17½-day stage was growth; both the maculae and the overlying otoliths had extended in area. The thickness of the layer of calcium salts had increased, whereas the gelatinous layer had become thinner, so that it was now no more than a membrane. The edges of the otolith were no longer diffuse.

The staining reactions of the parts of the otolith to haematoxylin and eosin were unchanged. When stained for phosphatase activity the granular matrix appeared greyish, and the gelatinous layer in some places slightly greyish, but otherwise negative. In the walls of the cavities no phosphatase activity could now be detected.

(*g*) *1 and 2 days.* In 1-day-old Bouin- and Susa-fixed animals the first sign of preservation of otolith material was noticed, and at 2 days a definite layer of granular matrix was preserved. The calcium salts and gelatinous layer were still not preserved.

(*h*) *3 days.* The two ears of one animal only were fixed in alcohol; one was stained with PAS and the other for phosphatase. No phosphatase activity was now present in either the gelatinous or the granular part of the matrix. The gelatinous layer took up the eosin counterstain and the granular matrix remained unstained. With PAS the granular matrix stained rose pink, while the gelatinous layer was doubtful. In the walls no phosphatase activity remained but in the lateral saccular wall some glycogen was still present.

One formalin-fixed ear was stained with the von Kossa calcium stain. Both otoliths gave a strong reaction, but otherwise there was no new feature.

The staining reactions of the granular matrix now preserved by Susa fixation were similar to those seen after formalin; with haematoxylin and eosin a blue colour, and with Masson's trichrome green. A strong positive reaction was obtained with the PAS stain.

Development of the cupulae and tectorial membrane

For the cupulae and the tectorial membrane, fixation with formalin or Bouin's fluid seemed to give a better picture of the state of affairs than alcohol fixation.

After all three fixatives the cupulae were already visible at 14½ days' gestation. After formalin or Bouin fixation they were found in the final normal position closely overlying the sensory epithelium of the cristae. After alcohol fixation they were displaced, towards the dorsal wall in the anterior and posterior ampullae, and either laterally or medially in the lateral ampulla. The cupulae stained green with Masson's stain, were PAS-positive, and stained black with cobalt sulphide after some hours' incubation for phosphatase. The cells of the cristae contained glycogen but no phosphatase. Other diffuse precipitated material was also present in the ampullae after alcohol fixation but appeared irrelevant, some probably being displaced from the utriculus, and some probably artefact. No significant change in the cupulae was noted after 14½ days.

The tectorial membrane was also visible at 14½ days after all three types of fixation. After Bouin fixation it appeared as a thin membrane lying in its final position, closely applied to the epithelium in the region of the future organ of Corti. After formalin fixation the appearance was similar, but in addition the cochlea was filled with a free precipitate with the same staining reactions as the tectorial membrane itself. After fixation with alcohol, on the other hand, the tectorial membrane was always moved from its normal position and displaced towards the vestibular membrane. In formalin- and Bouin-fixed material the tectorial membrane appeared to increase in thickness from 14½ to 17½ days' gestation, and by 17½ days the free precipitated material had disappeared from the cochlea of formalin preparations. Like the cupulae the tectorial membrane was PAS-positive and stained green with Masson's stain, but, unlike the cupulae, it gave no reaction for phosphatase.

Glycogen was present in the cells of the future vestibular membrane from 14½ to 18½ days' gestation, but there was none in the future stria vascularis. No phosphatase reaction was given by any part of the cochlear duct.

DISCUSSION

In attempting to formulate some hypothesis about the formation of the otoliths one must first consider the nature of the various substances, then their probable sites of origin, and then one may attempt to deduce chemical mechanisms involved in the deposition of these calcareous masses in the lumen of the sacculus and utriculus.

In this study of the mouse otolith the alcohol-fixed material has shown that the fully developed otolith consists of at least three parts: (*a*) the gelatinous layer, (*b*) the granular matrix, and (*c*) the inorganic salts.

The gelatinous layer had the appearance of a protein precipitate, and was not preserved by formalin, which is not a protein precipitant. It was therefore believed to consist of a globular protein and probably to be at least semifluid in life. No other definite properties, such as the presence of polysaccharides or phosphatase, were detected.

The granular matrix gave a positive reaction to the PAS stain and may therefore be presumed to contain a polysaccharide. Since it showed phosphatase activity protein was presumably present also. After formalin fixation this component of the otoliths was still present, but its staining reactions were rather different. This could mean either that the formalin had reacted with the granular matrix in such a way as to change its staining properties, or that some component of it had been preserved by one fixative and not by the other. It does not seem possible to decide between these alternatives, but it is well known that formalin can change the staining properties of proteins, so as to make them stain more strongly with basic dyes (Baker, 1945). Similarly, fixation with Bouin or Susa showed that the granular matrix became resistant to acid fixation at about 2 days after birth, and again one cannot say whether this was due to the secretion of a new component of the matrix, or to a change in the properties of substances secreted previously. However, the effect seen in pallid mice (described by Lyon, 1955), in which the otoliths fail to calcify, suggests that at least three layers of matrix are formed: at 15½–16½ days, 17½ days, and at 1–3 days after birth.

Recent work by Bélanger (1953) has shown by means of an autoradiographic study of the uptake of S^{35}, and by metachromasia after toluidine blue staining, that the otolithic membrane may be assumed to include a sulphur-containing mucopolysaccharide. It is reasonable to suppose that this substance was the polysaccharide seen in the granular matrix in the present work.

The calcium salts were detected by their birefringence. This was first seen as minute pin-points, and only later as granules of a definite shape. It seems likely that this pin-point material was not yet solid in life, but had been precipitated by the fixative, so that its localization may have shown the points of precipitation rather than the location of calcium salts in life. The appearance of birefringence coincided with the appearance of the mucopolysaccharide and increased in amount in parallel with it.

Very little evidence was obtained about the parts of the labyrinth wall which secreted the various parts of the otolith. In the alcohol-fixed material at the earliest stages the otolith material was lying near the lateral wall of the sacculus and the dorsal wall of the utriculus, in both cases the wall opposite the macula. However, it was clear from observation of the tectorial membrane and cupulae that alcohol fixation caused displacement of the epithelial coverings of the labyrinth, and in fact in some specimens otolith material had obviously been displaced by the alcohol from the utriculus into the ampullae. Hence, the localization seen after alcohol fixation must be considered unreliable. After formalin or Bouin fixation very little otolith material was seen until a stage in which even after alcohol fixation the newly formed otolith was lying near the macula; so the position of first appearance of the developing otolith remains unknown. Its apparent migration across the lumina with age in alcohol-fixed material could be due to a change with time in its resistance to displacement. However, in the 16½- and 17½-day alcohol specimens, some of the wisps of otolithic material and pin-points of birefringence touching the wall of the secretory regions of the utriculus presented a convincing appearance, and could be believed to have been there in life. Thus it is possible that some at least of the material taking part in the formation of the otolith was formed by the secretory regions of the utriculus wall. It seems more probable, however, that most of it was secreted by the maculae; the presence of phosphatase in the surface of these cells suggests some secretory function. In the sacculus the accumulation of glycogen in the lateral wall might indicate some histogenetic activity, but could also merely be associated with the poor blood supply of this region.

As it is not certain which cells secrete the various parts of the otolith, little can be said about the mechanism of its deposition. The widespread birefringence at 15½ days suggested that at this stage the endolymph had been rich in ionic calcium, which was precipitated by the fixative to form birefringent particles. What has not been brought out by the present work is the mechanism by which this enrichment occurred. It is not possible to say whether the calcium was actively secreted in an ionic form, or was split off in the lumen of the inner ear from some previously unionized compound, or whether, on the other hand, the ionic calcium concentration was raised by local absorption of water from the endolymph. The substances present, mucopolysaccharide, glycogen, phosphatase, and salts, are similar to those concerned in the calcification of bone and cartilage. In bone it is suggested that the glycogen is used to form hexose phosphates, from which the phosphate is liberated by alkaline phosphatase and fixed to the protein matrix, while calcium ions may be fixed to the mucopolysaccharide. There is then a chemical change in the organic matrix and the calcium and phosphate ions are liberated and precipitated as bone salt (Pritchard, 1952). In otolith formation the appearance and development of the mucopolysaccharide ran parallel to that of the calcium salts and the picture suggested that the mucopolysaccharide was important in calcification. On the other hand, the phosphatase activity was weak and it is doubtful whether it had any role in calcification, particularly since the salts of the otolith are chiefly calcium carbonate. Phosphatase has also been found during the laying down of calcium carbonate by invertebrates (Wagge, 1951) but here again its role was not clear. In the present work phosphatase was also present in the cupulae, which do not calcify, making it seem more probable that its function in cupulae and otoliths was to do with fibrillar protein secretion (Bradfield, 1949).

SUMMARY

1. The development of the otoliths in mice from 14½ days' gestation to 3 days after birth was studied in alcohol-, formalin-, and Bouin-fixed material.

2. The first sign of the otoliths in alcohol-fixed 14½-day material was an apparent protein precipitate. Then a PAS-reacting substance appeared among the protein, together with birefringent particles. Calcium salts were then deposited in their typical crystalline form and the otolith flattened so that at 16½ days it consisted of a gelatinous layer surmounted by a layer of birefringent calcareous granules with an organic matrix, which contained polysaccharide and phosphatase. Glycogen was also present.

3. After formalin fixation very little otolith material was seen until 16½ days, and with Bouin very little until 2 days after birth; formalin preserved the calcium salts and granular matrix, Bouin the granular matrix only.

4. Comparison of the results of the various fixatives showed that the localization seen after alcohol fixation was unreliable, so that the position of the otolithic material when it first appeared, and hence the site of its secretion, were unknown.

5. The cells of the maculae showed phosphatase activity and probably took

part in secreting substances; the secretory regions of the utriculus may also have done so.

6. The mechanism of concentration and precipitation of the calcium salts remains unknown, but it is suggested that the polysaccharide matrix may take part.

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REFERENCES

BAKER, J. R. (1945). *Cytological Technique*. London: Methuen & Co. Ltd.

BÉLANGER, L. F. (1953). Autoradiographic detection of S^{35} in the membranes of the inner ear of the rat. *Science*, **118**, 520–1.

BRADFIELD, J. R. G. (1949). Phosphatase cytochemistry in relation to protein secretion. *Exp. Cell Res. Supp.* **1**, 338–50.

CAMERON, G. R. (1930). The staining of calcium. *J. Path. Bact.* **33**, 929–55.

DALLEMAGNE, M. J. (1950). The physiology of supporting tissue. *Ann. Rev. Physiol.* **12**, 101–18.

GOMORI, G. (1939). Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. exp. Biol., N.Y.* **42**, 23–26.

GRÜNEBERG, H. (1943). The development of some external features in mouse embryos. *J. Hered.* **34**, 89–92.

HASTINGS, A. B. (1935). Chemical analysis of otoliths and endolymphatic sac deposits in *Amblystoma tigrinum*. *J. comp. Neurol.* **61**, 295–6.

HAZAMA, O. (1929). Die absondernden Zellelemente des Wirbeltierlabyrinths. *Z. Anat. EntwGesch.* **88**, 223–61.

IWATA, N. (1924). Über das Labyrinth der Fledermaus mit besonderer Berücksichtigung des statischen Apparates. *Aichi J. exp. Med.* **1**, No. 4, 41–173.

LORCH, I. J. (1947). Localization of alkaline phosphatase in mammalian bones. *Quart. J. micr. Sci.* **88**, 367–81.

LYON, M. F. (1951). Hereditary absence of otoliths in the house mouse. *J. Physiol.* **114**, 410–18.

—— (1953). Absence of otoliths in the mouse: an effect of the pallid mutant. *J. Genet.* **51**, 638–50.

—— (1955). The developmental origin of hereditary absence of otoliths in mice. *J. Embryol. exp. Morph.* **3**, 230–41.

MCMANUS, J. F. A. (1948). The periodic acid routine applied to the kidney. *Amer. J. Path.* **24**, 643–53.

NISHIO, S. (1926). Über die Otolithen und ihre Entstehung. *Arch. Ohr.-, Nas.-, u. KehlkHeilk.* **115**, 19–63.

PRITCHARD, J. J. (1952). A cytological and histochemical study of bone and cartilage formation in the rat. *J. Anat., Lond.* **86**, 259–77.

STOCK, A. (1949). The determination of calcium in histological sections. *J. R. micr. Soc.* **69**, 20–24.

TENAGLIA, G. (1925). La struttura degli otoliti osservati a fresco in campo oscuro. *Atti Soc. lombarda Sci. med. biol.* **14**, 188–94 (*Physiol. Abstr.* **10**, 541).

WAGGE, L. E. (1951). The activity of amoebocytes and of alkaline phosphatases during the regeneration of the shell in the snail, *Helix aspersa*. *Quart. J. micr. Sci.* **92**, 307–21.

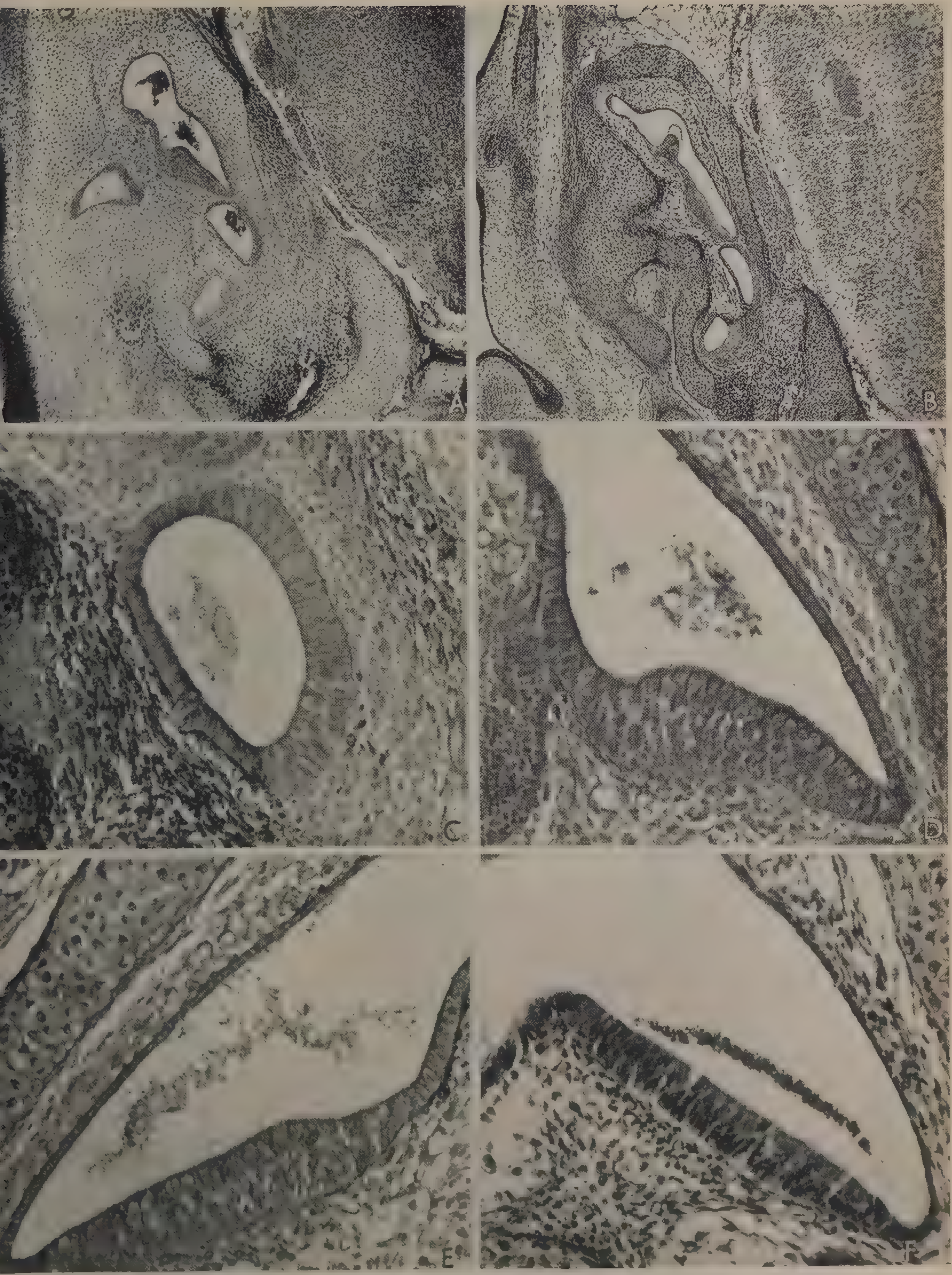

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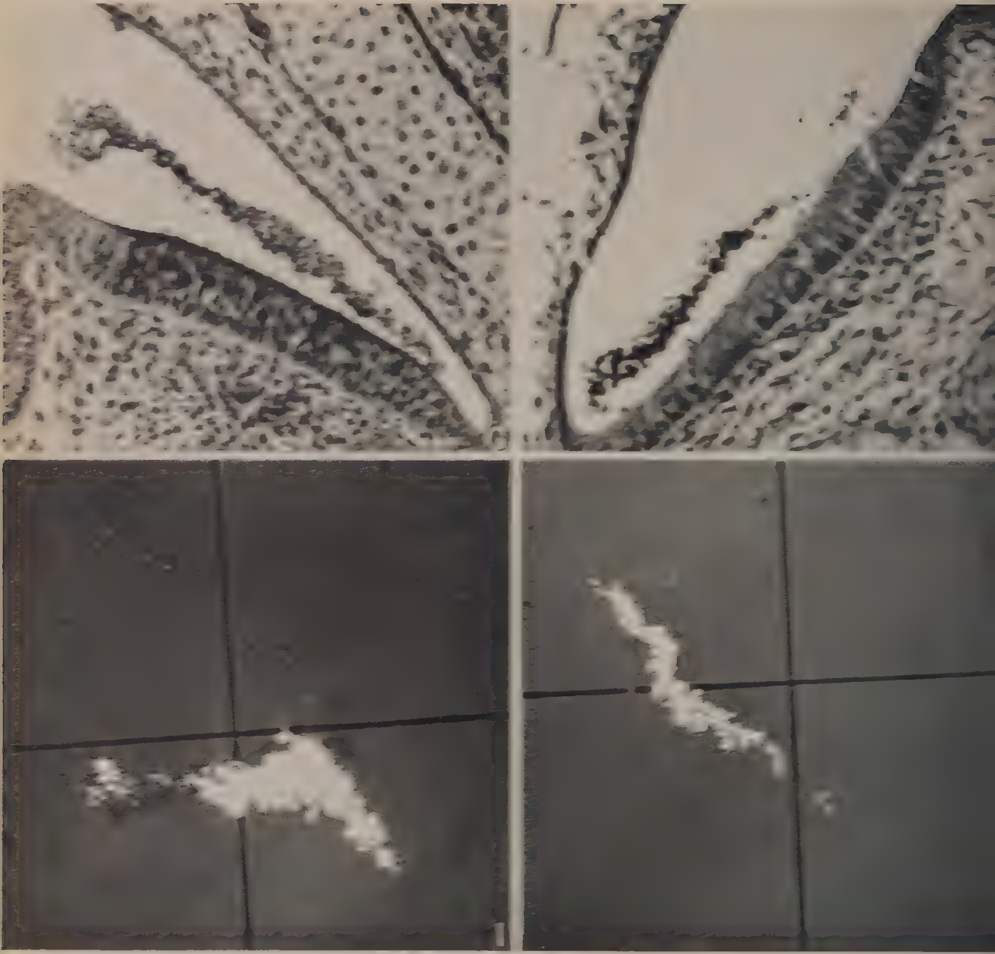
Plate 1



M. F. LYON

Plate 2

EXPLANATION OF PLATES

PLATE 1

FIG. A. Inner ear of 15½-day mouse embryo. Alcohol-fixed. Stained for phosphatase. ×55. The cupula, the otolith precipitate in sacculus and utriculus, and the surface of the cells of the maculae have reacted for phosphatase.

FIG. B. Formalin-fixed 15½-day embryo. PAS, light green. ×55. Only small specks of otolith material are preserved in sacculus and utriculus.

FIG. C. Alcohol-fixed 14½-day embryo. PAS, light green. ×230. Glycogen (dark) is visible in connective tissue, cartilage, lateral wall of the sacculus, and the otolith precipitate (specks only). The bulk of the otolith precipitate is negative to PAS (light).

FIG. D. Utriculus of alcohol-fixed 15½-day embryo. PAS, light green, amylase-treated. ×230. Glycogen having been removed, the PAS-reacting filaments in the otolith precipitate scarcely stand out from the background.

FIG. E. Utriculus of alcohol-fixed 16½-day embryo. PAS, amylase-treated. ×230. Granular matrix is present but its PAS staining is not sufficiently dark to stand out. Note wisps of matrix in contact with the pigmented regions of the wall.

FIG. F. Utriculus of formalin-fixed 16½-day embryo. PAS, haematoxylin. ×230. The PAS staining of the otolith granular matrix is much darker, but the gelatinous layer and wall wisps are missing.

PLATE 2

FIG. G. Utriculus of alcohol-fixed 17½-day embryo. Phosphatase. ×250. The otolith granules and cells of the macula have reacted for phosphatase.

FIG. H. Utriculus of formalin-fixed 17½-day embryo. Haematoxylin and eosin. ×250. No gelatinous layer of matrix is visible; the calcium salts have reacted strongly with haematoxylin.

FIG. I. Utriculus of alcohol-fixed 16½-day embryo. Seen by polarized light. ×210. The calcium granules are strongly birefringent; in addition there are pin-points of birefringence among the wisps of matrix near the pigmented regions of the wall.

FIG. J. Sacculus of alcohol-fixed 16½-day embryo. Seen by polarized light. ×210. Birefringence is visible in the calcium granules and (a few specks only) in the diffuse matrix at the edge of the otolith.

(Manuscript received 28 : x : 54)

The Developmental Origin of Hereditary Absence of Otoliths in Mice

by MARY F. LYON[1]

From the Institute of Animal Genetics, Edinburgh

WITH TWO PLATES

INTRODUCTION

HEREDITARY absence of otoliths was discovered by Lyon (1951) in mice homozygous for the mutant *pallid*. In transverse sections of 4-week-old mice the ears appeared normal except for the lack of otoliths.

The pallid mutant had been known previously for its effect on pigmentation: the coat colour is reduced to pale grey, and the eyes are pink. All animals homozygous for the mutant gene regularly show these pigment defects, but not all lack otoliths, and those that do show all grades of defect. Usually any particular otolith is either normal or completely absent, but occasionally otolith granules are present though fewer and larger than normal.

The present paper describes an investigation of the development of this otolith abnormality. It was hoped that such a study would provide some clue about the cause of the irregular manifestation of the defect, and of its association with reduced pigmentation. A study of the formation of otoliths in normal mice, carried out at the same time (Lyon, 1955), showed the presence of polysaccharide, phosphatase, and glycogen in the developing otolith matrix, and so the appearance of these was followed in pallid mice. In addition, the pigment normally present in the inner ears of mice was found to be absent from the ears of pallid mice, so the pigmentation of the ears of various other mutants was noted to find if there were any parallelism between pigment and ear abnormality. This point was of particular interest since pigment is regularly found in the inner ear of all vertebrates in the 'secretory regions' (certain regions of the utriculus and semicircular canals which from their histology are thought to have a secretory function). Hazama (1929), in pointing this out, suggested that this pigment must have some function. It is still not known whether this is true, although the association of genetic ear abnormalities with pigment defects has often been remarked on from the time of Darwin (1876), who pointed out that white cats

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with blue eyes are frequently deaf, to Waardenburg (1951), who described an inherited human syndrome including, among other things, white spotting and deafness, and who reviewed similar instances in cats, dogs, and mice.

MATERIALS AND METHODS

The animals used were all pallid homozygotes and came from a stock segregating for this gene. The normal animals of the same stock have been described previously (Lyon, 1955). Stages of 14½ to 18½ days' gestation and from birth to 4 days old were studied.

The methods used were the same as those described in the previous paper. Alcohol-, formalin-, and Bouin- or Susa-fixed specimens were stained with PAS or for phosphatase or calcium salts, or to show general ear morphology.

RESULTS

The first section describes the development of the otoliths of pallid mice by stages from 14½ days' gestation. Table 1 shows the material available. A separate section and table deal with the ear pigmentation of other mutants.

TABLE 1

The number of specimens, fixed and stained in various ways, which were available at each stage of development

		Stage in days									
Fixative	*Stain*	14½	15½	16½	17½	18½	*Birth*	1	2	3	4
Alcohol	Phosphatase	—	3	—	5	—	—	—	—	—	—
	PAS	5	3	6	2	—	—	—	—	1	—
	Birefringence	—	2	1	—	—	—	—	—	—	—
	Haematoxylin and eosin	—	1	—	—	—	—	—	—	1	—
Formalin	PAS	2	2	4	2	—	—	—	—	—	—
	Haematoxylin and eosin	—	1	—	7	3	—	—	—	—	—
	Masson or Mallory	2	5	6	6	—	—	—	—	—	—
	Kossa	—	2	2	1	—	—	—	—	1	—
Bouin or Susa	PAS	1	1	3	—	—	—	—	—	2	—
	Haematoxylin and eosin	—	—	1	3	—	18	8	7	4	2
	Masson or Mallory	2	—	—	1	—	2	6	9	20	7

Development of the otoliths of pallid mice

(1) *14½ days.* In no specimens were any differences from normal observed, either in the developing otoliths or in the labyrinth walls.

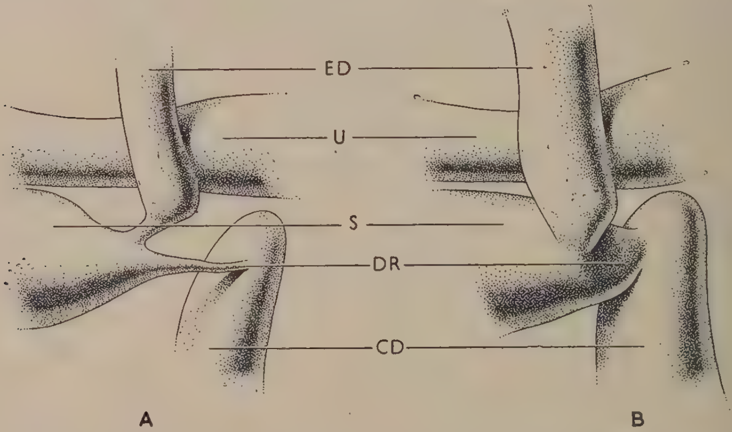
In the alcohol-fixed material a loose network of protein precipitate floated free in the sacculus and the utriculus. In addition, glycogen and membranous PAS-positive specks were present, and in the utriculus of one specimen there were a few PAS-positive granules. Birefringence was not tested.

(2) *15½ days.* Two alcohol-fixed specimens were tested for birefringence. Both showed none at all in the inner ear, though two normal sibs of one of these

animals showed widespread pin-point birefringence covering the matrix and cavity walls, and a pallid sib of the other gave a reaction for calcium at one point in the utriculus when tested with haematoxylin.

These specimens showed no difference from normal in the amount, position, or staining reactions of the developing otoliths (Plate 1, fig. A). Three other alcohol-fixed animals were tested for phosphatase. The otolith matrix and the walls of all the ears gave normal reactions. In some cases a few phosphatase-positive granules were visible among the loose matrix of the utriculus, and in other ears there were none. This variation was also seen among the normal sibs.

The walls of the sacculus and utriculus were normal in all specimens, whether alcohol-, formalin-, or acid-fixed, except in one respect: pigment was not visible in the secretory regions of the utriculus. These regions showed their normal rich blood-supply, but were quite unpigmented, though all normal animals of this age showed at least some pigment cells.

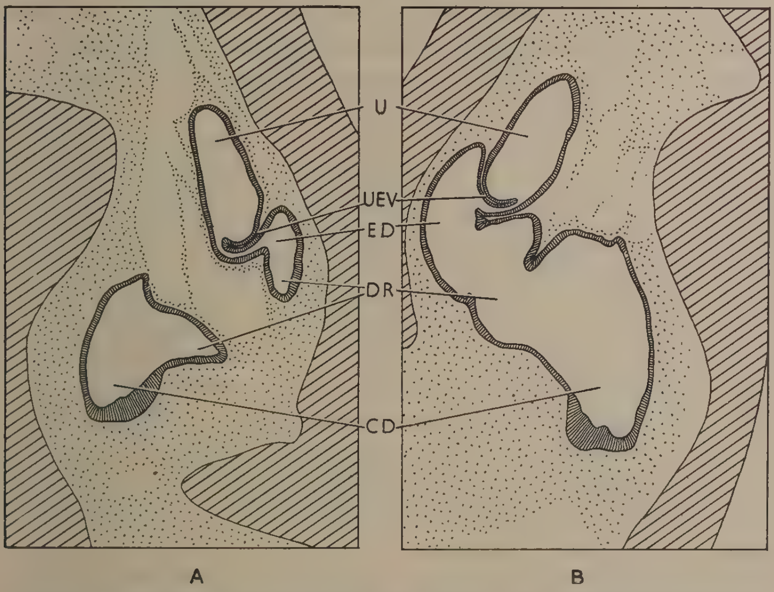


TEXT-FIG. 1. Reconstruction of the region of the ductus reuniens in two ears of 16½-day pallid embryos. The first (A) had normal otoliths and shows normal distension of the ear. The other (B) lacked otoliths and shows hydrops of the sacculus, cochlear duct, ductus reuniens, and endolymphatic duct, while the utriculus is unaffected. Reconstructed by projection of sections. ×85. *CD*, cochlear duct; *DR*, ductus reuniens; *ED*, endolymphatic duct; *S*, sacculus; *U*, utriculus.

(3) *16½ days.* Three alcohol-fixed animals were tested for birefringence. Two showed none at all, either in the otolith matrix or lining the walls, although normal sibs showed a layer of birefringent granules and surrounding pin-point birefringence. The third specimen showed normal birefringence and a layer of calcium granules in the right ear, but no birefringence and no granules in the left.

In all ears without birefringence and in one other alcohol-fixed specimen lacking otolith granules, the underlying gelatinous layer of the otolith was present. It was now lined up and forming a flat layer parallel to the macula, just as in the normal alcohol-fixed specimens of this age (Plate 1, fig. B). In addition, in the utriculus there were wisps of material in contact with the walls near or at the secretory regions and some in the mouths of the ampullae (displaced?). The other two alcohol-fixed animals had reached an earlier stage of development and the matrix was not yet lined up. Among formalin-fixed specimens some ears now showed a normal layer of PAS-positive granular matrix, and others lacked it.

An additional abnormality was now apparent in all alcohol- and formalin-fixed ears lacking calcium granules. This was a distension with fluid of the endolymphatic system. The sacculus, ductus reuniens, cochlear duct, and endolymphatic duct were all grossly enlarged, and the endolymphatic sac slightly so. The utriculus and semicircular canals, however, were of normal dimensions. Text-fig. 1 shows a reconstruction of the region in which the enlargement was most striking. Some acid-fixed ears also showed distension, but as no otolith material was preserved even in normal mice by this method of fixation, no other abnormality was visible.



TEXT-FIG. 2. Transverse section through the region of the utriculo-endolymphatic valve in the two ears of a 17½-day pallid embryo. The left ear (A) is normal and the right (B) is abnormally distended, but the utriculus and valve are normal in both ears. Camera lucida drawing. ×85. *CD*, cochlear duct; *DR*, ductus reuniens; *ED*, endolymphatic duct; *U*, utriculus; *UEV*, utriculo-endolymphatic valve.

(4) *17½ days.* Some alcohol- and formalin-fixed ears now possessed well-developed otoliths in the sacculus and utriculus, while others lacked them. As at 16½ days, all the abnormal ears showed endolymphatic hydrops, again in-

volving the cochlear duct, ductus reuniens, sacculus, and endolymphatic duct, while the utriculus and semicircular canals were unaffected. Text-fig. 2 gives a section through the utriculo-endolymphatic valve, showing that this too was normal.

In all alcohol-fixed ears without otoliths the gelatinous layer of matrix was lined up over the macula. It gave reactions with PAS and for phosphatase, and the cells of the maculae also reacted normally for phosphatase (Plate 1, fig. C). In addition, in some (but not all) of the abnormal ears there was a little free debris in the cochlear duct, sacculus, ductus reuniens, and endolymphatic duct. This material was phosphatase- and PAS-positive and appeared to be otolith matrix that had gone astray, since no phosphatase-containing material was normally found in the lumen of the cochlear duct. Some formalin-fixed ears which lacked otoliths also had a certain amount of free PAS-positive material distributed similarly, but no part of the otolith was found in its normal position, since formalin did not preserve the gelatinous layer (Plate 2, fig. D).

(5) *18½ days.* Only formalin-fixed specimens were studied.

In ears with no otoliths visible there were now no abnormal distension and no free material in the cochlear duct or sacculus. Instead the endolymphatic sac now contained a free precipitate, and its lumen was somewhat enlarged.

(6) *Newborn.* Only Susa-fixed animals were available.

No otoliths were preserved either in pallid mice or their normal sibs. Some pallids still showed some free material of variable staining in the lumen of the endolymphatic sac, especially posteriorly. In addition some animals, from two litters only, had a certain amount of precipitated material, which stained blue with haematoxylin, free in the lumen of the sacculus.

(7) *1 day.* In normal animals a little otolith matrix was now preserved, but so little that in pallids it was difficult to decide whether otoliths had been present or not. However, of ten ears which probably lacked otoliths eight had a considerable amount of precipitated material free in the lumen of the sacculus. Six also had similar material in the lumen of the endolymphatic sac, lying against its lateral wall. There was none in the utriculus. When stained, this free material appeared green after Masson's stain, and blue with haematoxylin and eosin. Wherever there was much in the endolymphatic sac, the lumen of this was distended.

(8) *2 days.* All the ears with no otolith matrix preserved had at least some debris. In some it was present in the sacculus and endolymphatic sac, in some in the endolymphatic sac only, and in two in the sacculus only. None was ever seen in the utriculus. All those endolymphatic sacs which contained free material had a wide lumen, while in the two ears which lacked otoliths but had no material in the endolymphatic sac this had a narrow lumen, as in the normal sibs and unaffected pallid ears.

(9) *3 days.* In this case alcohol-, formalin-, and acid-fixed specimens were available.

Again, all ears which lacked otoliths contained free precipitated material preserved by all three types of fixative. As at 2 days, some of the material lay in the sacculus and some in the endolymphatic sac, but there now seemed relatively more in the endolymphatic sac and less in the sacculus than at 2 days. As before, wherever there was much in the endolymphatic sac its lumen was distended (Plate 2, figs. F, G). In the sacculus the material floated freely (Plate 2, fig. E), but in the endolymphatic sac it lay against the lateral wall and appeared to have been formed into a compact mass. The total amount visible in the ear varied from specimen to specimen.

When stained the material proved PAS-positive and was negative to the von Kossa calcium stain.

In alcohol-fixed specimens there was still a layer, apparently corresponding to the gelatinous layer of normal mice, lying along the tips of the sensory hairs of the maculae. After other fixatives this layer was not visible.

In ears of pallid mice in which otoliths were present no free material was seen, and in fact, except for the absence of pigment from the secretory regions, no difference was detected from the ears of normal mice.

(10) *4 days.* Out of seven acid-fixed ears of pallid mice lacking otoliths six had some material in the lumen of the endolymphatic sac. None had any in the sacculus.

Pigmentation of the inner ear of mouse mutants

The inner ears of mice carrying various mutant genes were fixed in Susa, cleared in methyl benzoate, and examined as whole mounts. The pigment of the secretory regions could then be clearly seen. Table 2 shows the results obtained.

TABLE 2

Pigmentation of the inner ear of mouse mutants

Mutant	*No. of specimens*	*Ear pigment*	*Hearing and behaviour*
c	15	Absent	Normal
p	10	Absent	Normal
b	10	Brown	Normal
v	10	Normal	Deaf, waltzer
kr	10	Present	Deaf, waltzer
Va	10	Normal	Deaf, waltzer

The first three mutants listed affect the pigment of the coat and eyes but not hearing or behaviour. In animals homozygous for *c*, albinism, the coat is white and the eyes pink; in *pp* animals the black pigment of the coat is reduced to pale grey while the yellow is unaffected, and the eyes are again pink. In the mutant *b*, brown, the black pigment of coat and eyes is replaced by brown. In all these three colour mutants the pigmentation of the ears paralleled that of the eyes. In *bb* mice the ear pigment was brown instead of black, and in *cc* and *pp* no ear pigment was visible. Yet the hearing and behaviour were unaffected.

The other three mutants affect hearing and behaviour. Varitint-waddler, *Va* (Cloudman & Bunker, 1945), also affects the pigmentation of the coat: over the greater part of the coat the black pigment is reduced to grey, but there are patches of normal intense colour and of white. This mutant produces no gross structural abnormality of the ear and the pigmentation was quite normal. Similarly, the structure of the ear of waltzer, *v*, is macroscopically normal, and again the pigment was normally distributed. Kreisler, *kr*, causes gross malformation of the inner ear, including absence of canals (Hertwig, 1944), and hence the pigment could not be distributed normally, but none the less it was clearly present.

Investigations were also made in a stock of spotted mice, in which the two mutants W^v and *s* were segregating. The mutant *s*, piebald spotting, produces a piebald effect when homozygous; W^v, viable macrocytic anaemia or dominant spotting, when heterozygous produces a little white spotting and a slight dilution of coat colour, and when homozygous results in a black-eyed-white phenotype.

TABLE 3

Correlation between ear pigment and behaviour in black-eyed-white mice

Behaviour	*Ear pigment*			
	None	*Partial*	*Normal*	*Total*
Choreic . .	23	3	1	27
Normal . .	25	12	4	41

The compound $W^v + ss$ may also be a black-eyed-white, or may have small areas of pigment round the pinnae and on the rump. In the particular stock investigated here some $W^v + ss$ animals showed choreic behaviour. This defect included rapid head-shaking, hyperactivity, and occasional circling, and some animals were apparently deaf. All the affected animals were of the black-eyed-white phenotype, but not all black-eyed-whites were affected. The genetic requirements for the abnormality therefore presumably included the presence of the $W^v + ss$ genotype and of a particular combination of modifying factors. The anatomical basis was not investigated in detail, but ears were examined to detect any association between absence of ear pigment and chorea. Sixty-eight black-eyed-white animals from this stock were examined. Some lacked ear pigment entirely, in some only parts of the secretory regions were pigmented, and in others a normal amount of pigment was present. There was, however, no clear association between pigment and chorea (Table 3). In fact, twenty-five animals which lacked pigment entirely showed completely normal hearing and behaviour. Thus, the ears of these animals could function quite normally when all pigment was absent.

DISCUSSION

In the abnormal ears all parts of the developing otoliths could be detected except the calcium salts.

The gelatinous layer of the matrix appeared normally, in the same relative position and amount as in normal mice, and with the usual staining reactions. The walls of the labyrinth also gave the normal reactions for phosphatase and glycogen, and differed only in the absence of pigment from the secretory regions.

Whether the granular matrix was entirely normal, however, is difficult to say. In normal mice it first appeared with the calcium salts at about 15½ days' gestation. Phosphatase and PAS-reaction, which are its characteristics, certainly appeared in abnormal ears at this stage but the gelatinous layer may have had these properties too. Later, at 17½ and 18½ days' gestation, in alcohol- and formalin-fixed specimens, and later still at 1–3 days after birth with all types of fixation, the sacculus of abnormal ears definitely contained some free material which gave the same reactions as granular matrix. Hence, it is only at the earliest stages that there is any doubt about the presence of this matrix, at least in the sacculus.

The effect seen in ears lacking otoliths confirms the impression, gained from normal mice, that the granular matrix was formed over an extended period. In normal mice at 15½ and 16½ days only a small, rather formless remainder of each crystal was left behind when the calcium salts were dissolved. After 17½ days the shape of the granules was preserved when the calcium was removed, and from 2 days after birth the matrix was resistant to acid fixation. In pallid mice little granular matrix was visible until debris appeared in the sacculus at 17½ days. This material passed to the endolymphatic sac and little more was seen until 1–2 days after birth, when a considerable amount of free acid-resistant matrix was found in the sacculus. Thus it seems that matrix continues to form from 15½ to 17½ days' gestation and again for a few days after birth.

The visible distension with fluid of the abnormal ears followed the failure of appearance of calcium salts. The distension was first detected at 16½ days, while in normal ears birefringence, due to calcium salts, was widespread at 15½ days. Hence it does not seem plausible to consider this hydrops as a cause of the failure of calcification. It is possible either that the hydrops was the result of the absence of calcium salts, or that both had a common cause.

The distension must have been due to some temporary disturbance of the fluid balance of the ear. Failure of deposition of calcium salts could also either be due to, or itself result in, a disturbance of fluid balance. Hence, one must consider the normal flow of endolymph. Aldred, Hallpike, & Ledoux (1940) showed that the endolymph of the cat is normally hypertonic with respect to blood and cerebro-spinal fluid, which is confluent with the perilymph. The stria vascularis of the cochlear duct and the secretory regions of the utriculus and canals are generally considered to secrete at least part of the endolymph, but it is not known to what extent the hypertonicity is due to active secretion. Guild (1927) showed that the endolymphatic sac took part in absorbing endolymph, and therefore Aldred, Hallpike, & Ledoux postulated that it performed work in actively absorbing water against an osmotic gradient, and that new fluid continually entered the endolymph from the perilymph through the walls of the labyrinth.

Further elucidation of the details of endolymphatic fluid balance has been complicated by two facts. First, that the two main parts of the labyrinth, the pars inferior and the pars superior, which are separated in mammals by the utriculo-endolymphatic valve (Roberts, 1932), are to some extent independent in water balance, and second, that there appear to be species differences among mammals. The first point is made clear by cases in which hydrops or collapse of the labyrinth is confined to the pars inferior only. The hydrops is seen in Ménière's disease of man, in which the cochlear duct, sacculus, and endolymphatic duct are swollen, while the utriculus and canals are normal (Lempert, Wolff, Rambo, Wever, & Lawrence, 1952) and also in the abnormal pallid embryos of the present work. Collapse of the cochlear duct and sacculus only, following atrophy of the stria vascularis and organ of Corti, occurs in congenitally deaf cats and dogs (Howe & Guild, 1933; Lurie, 1948) and in some human deaf mutes (Gilse, Hinnen, & Nieuwenhuyse, 1942). The utriculo-endolymphatic valve must be important in maintaining this independence. Species differences are suggested by apparently conflicting results obtained in different animals. Guild's (1927) original observations on the absorptive function of the endolymphatic sac were made on guinea-pigs. Lindsay (1947) and Lindsay, Schuknecht, Neff, & Kimura (1952), working with monkeys and cats respectively, tried to demonstrate this function by destroying the endolymphatic sac, but failed to obtain the distension with fluid that they expected. On the other hand, genetic absence of the endolymphatic sac in two mouse mutants, kreisler, *kr*, and dreher, *dr*, is associated with gross distension of the endolymphatic system (Hertwig, 1944, 1951). Similarly, although, as quoted above, atrophy of the stria vascularis in cats and dogs is accompanied by collapse of the cochlear duct and sacculus, a similar atrophy in waltzing guinea-pigs (Lurie, 1941) and in shaker-1 mice (Grüneberg, Hallpike, & Ledoux, 1940) leaves the labyrinth normally distended. These results suggest that in rodents the stria vascularis is less and the endolymphatic sac more important than in carnivores and primates.

In the case of absence of otoliths the hydrops is only temporary, so there is no reason to suspect any permanent abnormality of the structures maintaining fluid balance. In fact, the apparent movement of free otolith material from the sacculus to the endolymphatic sac in abnormal pallid embryos strongly suggests that the endolymphatic sac was functioning normally.

It is thus necessary to look for some temporary disturbance which will account for both the failure of deposition of calcium salts and the hydrops. Various explanations could fit the facts observed. One possibility is that a normal secretion of calcium into the endolymphatic lumen does not occur. Then one would have to suppose the abnormal distension to be due to other substances which remain in solution and increase the osmotic pressure. For example, the endolymphatic sac was distended when free matrix was present; it is thus conceivable that the matrix was exerting some osmotic effect. Alternatively, the calcium may pass normally into the endolymphatic lumen but not be deposited. Possible mechanisms for this include, first, a temporary failure of absorption of water so that the calcium was not sufficiently concentrated and, in addition, swelling resulted. This would be plausible if some part of the labyrinth wall had the temporary function of concentrating the endolymph at the time of calcification of the otoliths and failed to discharge this function. Secondly, it might be that the concentration of calcium in the endolymph was normal, but some mechanism failed which normally causes the calcium to be held and deposited with the matrix. Then the presence of calcium ions in solution would raise the osmotic pressure and lead to abnormal distension with fluid. Perhaps absence of matrix at the earliest stages, or some disturbance of pH, could cause such an effect.

It remains unexplained why no free matrix was seen in the utriculus of mice lacking otoliths and why the utriculus and canals were never hydropic. It is possible that the matrix for the utricular otolith was never formed in an abnormal ear. But a preferable hypothesis is that the failure to observe both matrix and hydrops was due to the same property of the normal endolymph flow which caused restriction of hydrops or collapse to the pars inferior in the other cases quoted.

Thus more detailed knowledge of the cause of failure of calcification of the otoliths in pallid mice must await better understanding of the normal development of the otoliths.

Another question which remains unsolved is the association between pigment defect and absence of otoliths in pallid mice. It is tempting to conclude that the absence of normal pigment from the secretory regions results in the lack of otoliths. However, albino and pink-eyed mice also have unpigmented secretory regions, and yet their inner ears function normally. Similarly, black-eyed-white spotted mice, $W^v + ss$, may lack ear pigment entirely and yet show no sign of abnormality in hearing or behaviour. This last case is particularly interesting since Silvers (1953) has reported that not only melanin granules but pigment cells themselves are entirely lacking from the white areas of the coat in spotted mice, whereas in albinos the cells are present but contain no melanin. It is of course possible that the pigment cells of pallid mice produce some abnormal substance which actively inhibits otolith formation, but since study of normal mice has shown that pigmentation of the secretory regions is only just beginning at 15½ days when the calcium salts of the otoliths appear, this does not seem a probable explanation.

SUMMARY

1. Hereditary absence of otoliths occurs in some mice homozygous for the mutant *pallid*. The development of the ears of pallid mice, with and without otoliths, was compared with that of normal mice.

2. In ears lacking otoliths all parts of the developing otoliths could be detected, except the calcium salts. Abnormality was first detected at 15½ days' gestation, when the normal birefringence due to calcium salts did not appear; from 16½ days some ears of pallid mice showed a normal layer of calcium granules while others lacked it.

3. The gelatinous layer of the otolith matrix appeared at the normal time, and was normal in amount, position, and staining reactions.

4. The granular matrix was not clearly present at the earliest stages, 15½ and 16½ days, at which it is normally seen. From 17½ days, however, abnormal ears contained free debris which, from its staining reactions, was interpreted as granular matrix. This debris was found in the sacculus and later the endolymphatic sac.

5. At 16½ and 17½ days' gestation ears lacking otoliths showed hydrops of the cochlear duct, sacculus, and endolymphatic duct.

6. Possible mechanisms for the failure of calcification and for the hydrops are discussed.

7. All ears of pallid mice lacked pigment from the secretory regions.

8. The ears of six other mouse mutants, some affecting pigment, and others affecting hearing and behaviour but not pigment, were examined for pigmentation. The pigment of the ear paralleled that of the eyes, and there was no correlation between lack of ear pigment and presence of behaviour defects.

ACKNOWLEDGEMENTS

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REFERENCES

Aldred, P., Hallpike, C. S., & Ledoux, A. (1940). Observations on the osmotic pressure of the endolymph. *J. Physiol.* **98,** 446–53.

Cloudman, A. M., & Bunker, L. E. (1945). The varitint-waddler mouse. A dominant mutation. *J. Hered.* **36,** 259–63.

Darwin, C. (1876). *The Origin of Species,* 6th ed. London: John Murray.

Gilse, P. H. G. van, Hinnen, A. B., & Nieuwenhuyse, A. (1942). Heredity in diseases in the field of oto-, rhino-laryngology. *Bibliogr. genet.* **13,** 301–424.

Grüneberg, H., Hallpike, C. S., & Ledoux, A. (1940). Observations on the structure, development and electrical reactions of the internal ear of the shaker-1 mouse (*Mus musculus*). *Proc. roy. Soc.* B, **129,** 154–73.

Guild, S. R. (1927). The circulation of the endolymph. *Amer. J. Anat.* **39,** 57–81.

Hazama, O. (1929). Die absondernden Zellelemente des Wirbeltierlabyrinths. *Z. Anat. EntwGesch.* **88,** 223–61.

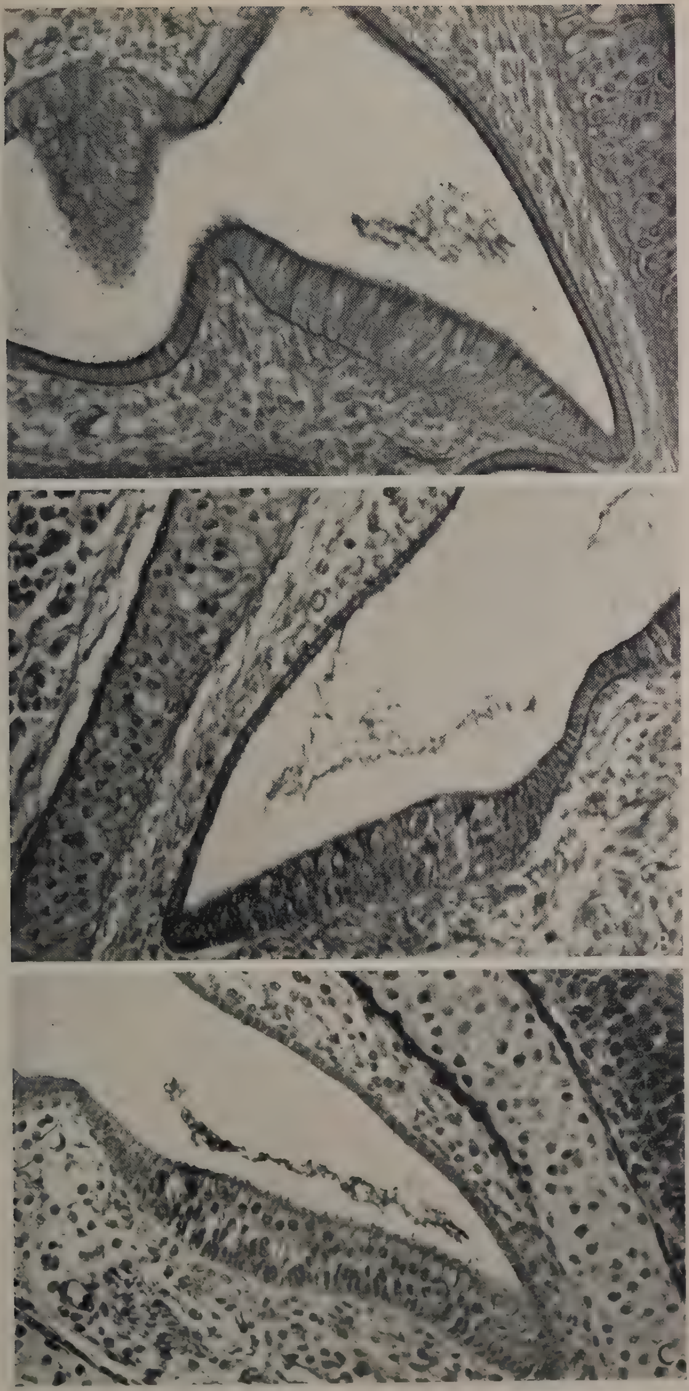

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Plate 1

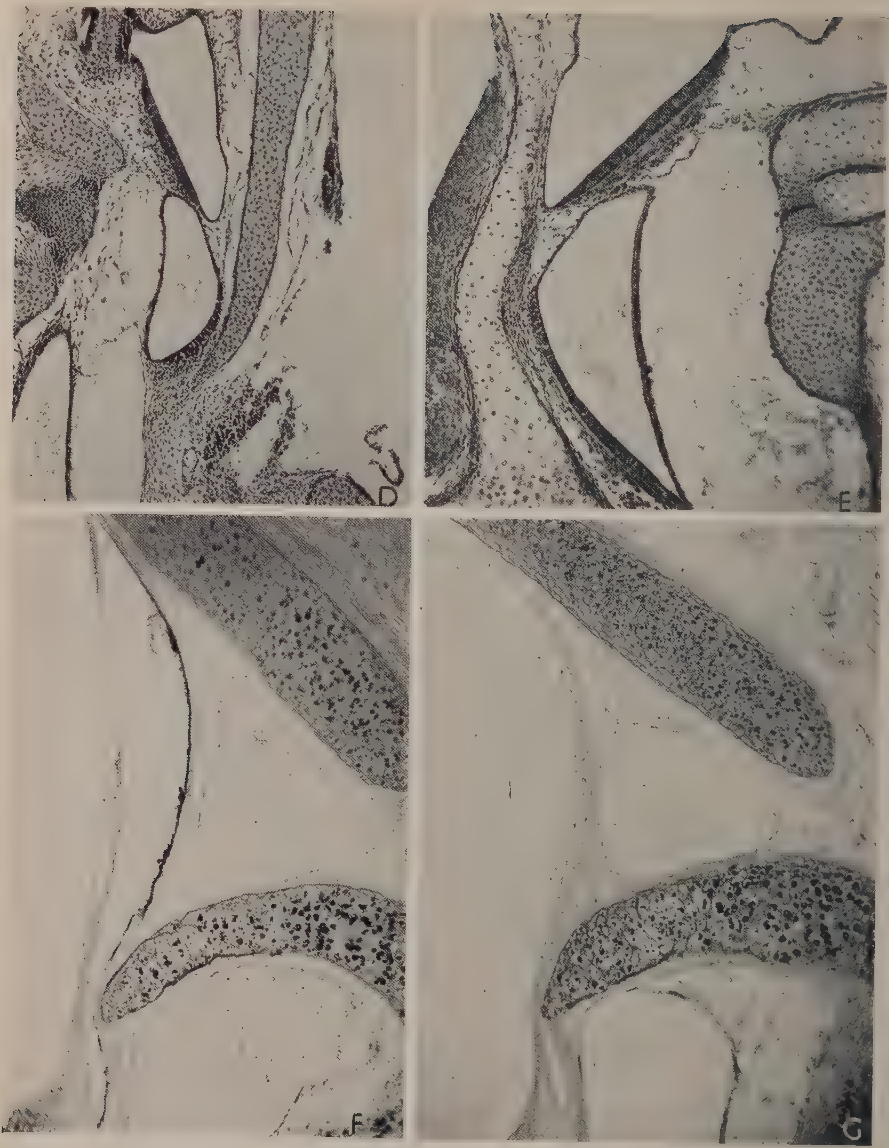

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Plate 2

HERTWIG, P. (1944). Die Genese der Hirn- und Gehörorganmissbildungen bei röntgenmutierten Kreisler-Mäusen. *Z. ges. Anat. 2. Z. KonstLehre*, **28,** 327–54.

—— (1951). Entwicklungsgeschichtliche Untersuchungen über Bewegungsstörungen bei Mäusen. *Verh. anat. Ges. Jena*, **49,** 97–107.

HOWE, H. A., & GUILD, S. R. (1933). Absence of the organ of Corti and its possible relation to electric auditory nerve responses. *Anat. Rec.* **55**, Abstr. 20–21.

LEMPERT, J., WOLFF, D., RAMBO, J. H. T., WEVER, E. G., & LAWRENCE, M. (1952). New theory for the correlation of the pathology and the symptomatology of Ménière's disease. *Ann. Otol., etc., St. Louis*, **61,** 717–37.

LINDSAY, J. R. (1947). Obliteration of the endolymphatic sac and duct in the monkey. *Arch. Otolaryng., Chicago*, **45,** 1–13.

—— SCHUKNECHT, H. F., NEFF, W. D., & KIMURA, R. S. (1952). Obliteration of the endolymphatic sac and the cochlear aqueduct. *Ann. Otol., etc., St. Louis*, **61,** 697–716.

LURIE, H. M. (1941). The waltzing (circling) guinea-pig. *Ann. Otol., etc., St. Louis*, **50,** 113–28.

—— (1948). The membranous labyrinth in the congenitally deaf collie and Dalmatian dog. *Laryngoscope, St. Louis*, **58,** 279–87.

LYON, M. F. (1951). Hereditary absence of otoliths in the house mouse. *J. Physiol.* **114,** 410–18.

—— (1955). The development of the otoliths of the mouse. *J. Embryol. exp. Morph.* **3,** 213–29.

ROBERTS, J. T. (1932). On the utriculo-endolymphatic valve in the albino rat. *Anat. Rec.* **53,** 255–64.

SILVERS, W. K. (1953). Histological distinction between hair follicles of albino and spotted genotypes of the mouse. *Genetics*, **38,** 691.

WAARDENBURG, P. J. (1951). A new syndrome combining developmental anomalies of the eyelids, eyebrows and nose root with pigmentary defects of the iris and head hair and with congenital deafness. *Amer. J. hum. Genet.* **3,** 195–253.

EXPLANATION OF PLATES

PLATE 1

FIG. A. Utriculus of alcohol-fixed 15½-day pallid embryo. PAS-light green, amylase-treated. ×250. The otolith precipitate shows no differences from normal.

FIG. B. Utriculus of alcohol-fixed 16½-day pallid embryo. PAS-light green, amylase-treated. ×250. The gelatinous layer of matrix has become lined up but no calcium granules have formed. Wisps of matrix are in contact with the utricular walls.

FIG. C. Utriculus of alcohol-fixed 17½-day pallid embryo. Phosphatase. ×250. The gelatinous layer is lined up and reacts for phosphatase, but calcium granules are absent. No pigment is visible in the walls.

PLATE 2

FIG. D. Utriculus, sacculus, and cochlear duct of 17½-day formalin-fixed pallid embryo. Haematoxylin and eosin. ×70. There are no otoliths but free debris is visible in the sacculus and cochlear duct, and, to a lesser extent, in the utriculus. The cochlear duct is enlarged.

FIG. E. Sacculus and utriculus of 3-day-old Susa-fixed pallid mouse. Masson's. ×100. Otoliths are absent. Free debris is present in the sacculus but not in the utriculus.

FIG. F. Endolymphatic sac of 3-day-old Susa-fixed pallid mouse. PAS. ×100. PAS-reacting debris lines the lateral wall. The lumen is distended.

FIG. G. Endolymphatic sac of a normal sib of the mouse of figure F, showing the normal collapsed lumen. ×100.

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Studies on the Development of the Foregut in the Chick

III. The Role of Mitosis

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INTRODUCTION

VENTRAL closure of the foregut in the chick embryo is preceded by a thickening of the presumptive foregut floor and a thinning of the presumptive foregut roof (Bellairs, 1953*a*). The present paper is concerned with quantitative estimates of the mitotic rates which may bear on the manner in which these changes are brought about.

MATERIAL AND METHODS

Ten specimens have been used in this investigation (see Table 1). Four were at the head process stage, 2 at the head fold stage, 3 were embryos with 4–5 pairs

TABLE 1

Description of specimens

Stage	*Specimen No.*	*Area pellucida length, mm.*	*Head process length, mm.*	*Primitive streak length, mm.*	*No. of somite pairs*
Head process	1	3·0	0·15	2·2	0
	2	3·3	0·3	2·2	0
	3	3·0	0·3	2·0	0
	4	3·4	0·4	2·3	0
Head fold	5	3·0	0·7	1·9	0
	6	3·5	0·8	2·0	0
			Head fold length, mm.	*Rest of embryo length, mm.*	
Somite	7	3·7	0·5	3·2	5
	8	3·5	0·65	2·8	4
	9	4·0	0·6	3·0	4
	10	6·5	1·7	4·7	12

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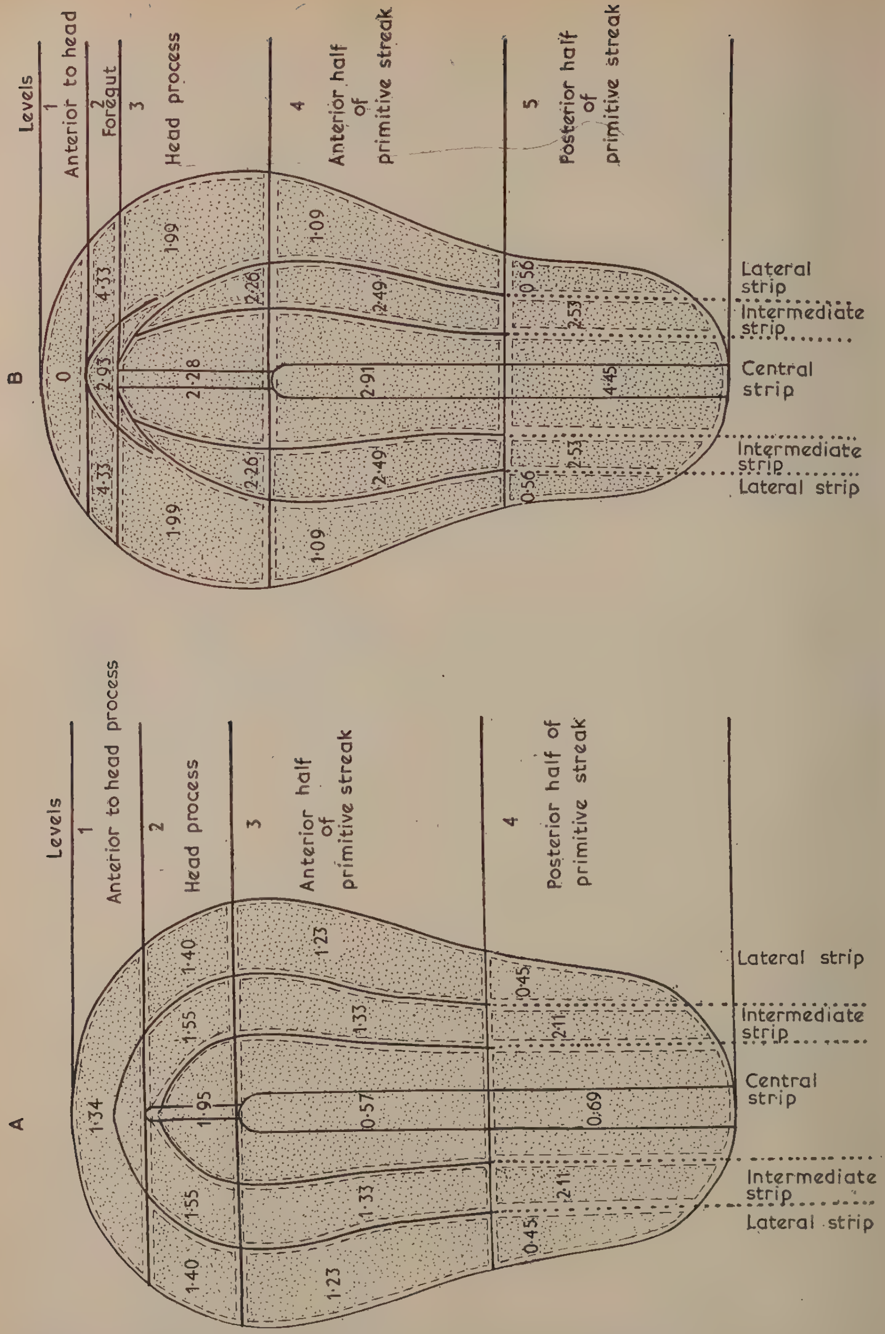
of somites, and the remaining embryo had 12 pairs of somites. Each specimen was removed from the yolk, which took about 2 minutes, and fixed immediately in Bouin's fluid. It was subsequently cut into serial sections at 7μ and stained with either Feulgen's solution and light green, or with Weigert's haematoxylin without counterstain.

Estimations of the mitotic rate were made on every third section throughout the whole area pellucida endoderm. Three longitudinal strips of endoderm were distinguished in each blastoderm as shown in Text-fig. 1, using criteria given below: *Central strip* (roof and/or presumptive roof of foregut, and midline endoderm posterior to the presumptive foregut region), *intermediate strip* (floor and/or presumptive floor of foregut, and the endoderm lying immediately on either side of the central strip in the posterior part of the area pellucida), and the *lateral strip* (extra-embryonic area pellucida endoderm). In levels where the endoderm was not folded into a foregut (i.e. throughout the head process stage specimens, and posterior to the anterior intestinal portal in the later stages) the intermediate endoderm and the lateral endoderm were each present as two separate portions; in such cases the results obtained for the two portions were pooled in every section.

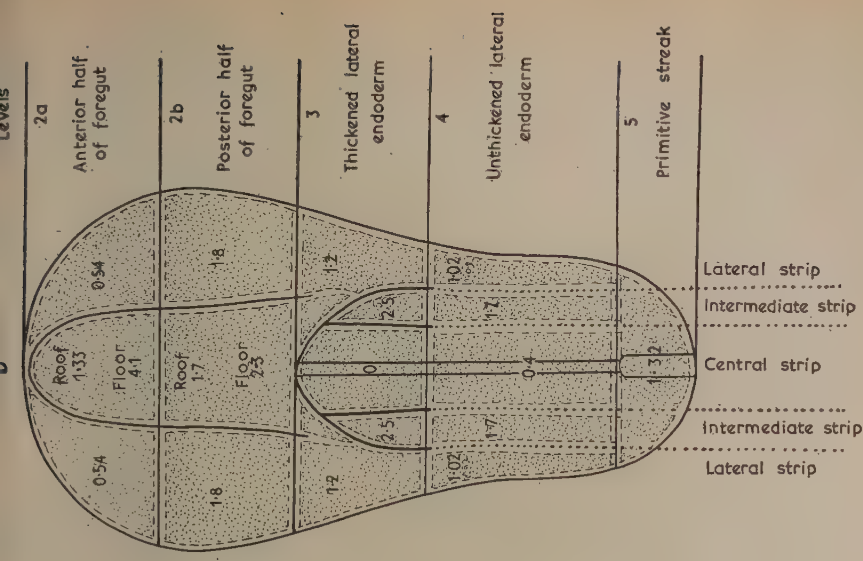
Every third section was studied at a magnification of 500 × with an oil-immersion lens. The mitotic counts were recorded for each region in phases; the mitotic rate was taken to be (number of dividing cells/number of resting cells) × 100. The counts of both resting and dividing cells obtained for the central, intermediate, and lateral strips were then grouped into levels (see Text-fig. 1) according to the antero-posterior position of the sections in the blastoderm. The levels were determined on morphological grounds.

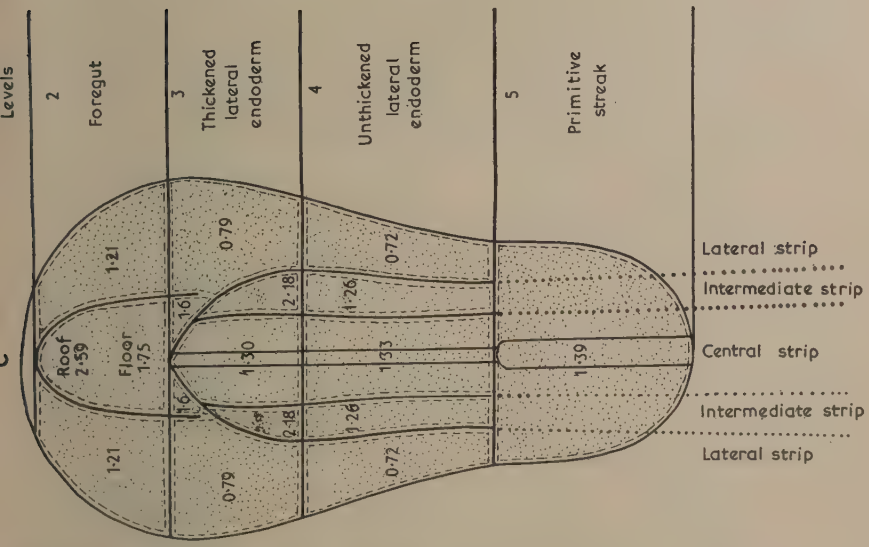
The χ^2 test was used for estimating the statistical significance of the differences in mitotic rate between different regions in each specimen; a probability of 5 per cent. was used as the level of significance.

Criteria for distinguishing between different regions. At the anterior end in the three most advanced stages the thin endoderm of the central strip (roof or presumptive roof) is easily recognized, for it is sharply demarcated at each side from the thick intermediate strip (floor or presumptive floor). The latter may also be distinguished on its distal side from the relatively thin lateral strip (extra-embryonic endoderm). These morphological differences gradually become less apparent further posteriorly in each specimen. It is also difficult to fix a limit to each of the chosen areas when the changes in thickness are only just beginning to develop, i.e. in the head process stage, or in the more posterior regions of older embryos. Another criterion which has also been employed therefore is the relationship which exists between the different regions of the endoderm and the overlying mesoderm. The junction between the central and the intermediate endoderm corresponds with the position of the developing intermediate cell mass; and the presumptive foregut floor endoderm is usually overlain by rather thickened splanchnic mesoderm. At certain levels, however (e.g. at the primitive



TEXT-FIG. 1. Diagram representing the ventral aspect of the endoderm of the four stages: A, head process; B, head fold; C, embryo with four pairs of somites; D, embryo with 12 pairs of somites. Each is considered as a number of antero-posterior levels and medio-lateral strips, whose limits are based on morphological features of the endoderm or of the overlying mesoderm. The endoderm is thus represented as a number of regions, the position of each being determined by the





tero-posterior level and the medio-lateral strip in which it lies. These regions are outlined by oken lines. Where the foregut has begun to form, as in B, C, and D, the roof is obscured in the agram by the floor, but its outline is indicated. In practice the floor itself is covered ventrally in is region by the (originally lateral) extra-embryonic endoderm which is continuous from side side beneath the foregut. For convenience, however, this extra-embryonic endoderm is not presented on the diagram but its mitotic rate is included in that of the lateral strips. In the head ld stage (B) the roof and floor of the actual foregut have been treated as one region. The mitotic tes are given for 4 representative specimens. A, specimen 3; B, specimen 6; C, specimen 8, D, specimen 10.

streak level of the somite stages), where it has been impossible to assess with any confidence the limits of the central, intermediate, and lateral endoderm, the overall mitotic rate has been estimated for the whole width of the endoderm.

RESULTS

The mitotic figures recorded were usually in the following proportions: prophase 16–22 per cent., metaphase 40–50 per cent., anaphase 17–19 per cent., telophase 16–19 per cent. The mitotic rates for the area pellucida endoderm as a whole tended to be higher in the early stages than in the later ones. There was, however, considerable variation between individuals; one of the early somite stage embryos, for example, was found to have a mitotic rate significantly higher than either of the other two. The over-all mean rates obtained were: head process stage, 2·44, 2·82, 1·26, and 1·28 per cent.; head fold stage, 1·59 and 1·53 per cent.; early somite stage, 0·56, 1·53, and 0·96 per cent.; and later somite stage, 1·53 per cent. Average figures have therefore not been drawn up for each age

TABLE 2

The mitotic rates expressed per 100 intermitotic cells obtained in the different regions of each of the four somite stage embryos. The number of dividing cells is given in brackets in each case. Total is the overall rate for the whole area pellucida

Specimen	*Level*	*Lateral strip*	*Intermediate strip*	*Central strip*	*Total*
7	2	0·35 (5)	2·00 (11)	0·53 (1)	0·56
	3	0·16 (2)	0·43 (7)	0·00 (0)	
	4	0·42 (5)	0·56 (7)	0·00 (0)	
	5	0·76 (21)			
8	2	1·21 (5)	1·75 (25)	2·59 (5)	1·53
	3	0·79 (20)	2·18 (60)	1·30 (9)	
	4	0·72 (10)	1·26 (14)	1·33 (7)	
	5	1·39 (38)			
9	2	0·40 (6)	0·94 (12)	1·10 (4)	0·96
	3	0·75 (13)	1·60 (24)	1·54 (6)	
	4	0·48 (10)	1·09 (7)	0·92 (4)	
	5	0·45 (12)			
10	2a	0·54 (4)	4·11 (15)	1·33 (5)	1·53
	2b	1·79 (11)	2·31 (42)	1·71 (3)	
	3	1·23 (8)	2·54 (14)	0·00 (0)	
	4	1·03 (10)	1·73 (20)	4·14 (1)	
	5	1·32 (20)			

group of embryos; attention has been concentrated rather on the differences which exist in the mitotic rate between different parts of the endoderm in each individual. Text-fig. 1 shows the mitotic rate in the endoderm of four specimens taken one from each group. Table 2 is of the mitotic figures obtained in the various parts of the endoderm in each of the four somite stage specimens.

The *head process specimens* had no region in which the mitotic rate was significantly higher than in any other, with one exception; in specimen 2 levels 2 and 3 of the central strip each had a mitotic rate which was significantly higher than that of either the intermediate or lateral strips at the same levels. Except at its anterior tip, this central strip is destined to become thinner; the high mitotic rate in this instance cannot therefore be associated with a thickening of the endoderm.

The main result obtained with the *older specimens*, where variations in thickness were now apparent, was that the mitotic rate in either the thick floor or the thickening presumptive floor was significantly raised. In 4 of the 6 specimens the total mitotic rate of levels 2, 3, and 4 of the intermediate strip was significantly higher than the over-all mitotic rate in the rest of the embryonic and extra-embryonic area pellucida endoderm. In all of the 6 specimens moreover, the intermediate endoderm at one or more levels was found to have a mitotic rate significantly higher than that in the lateral endoderm at the same level.

Both of the *head fold stage* specimens had a mitotic rate in level 4 of the intermediate strip which was significantly higher than that in the same level of the lateral endoderm, although but little thickening was apparent. In specimen 5, however, the thick intermediate endoderm of the closed gut at level 2 had a mitotic rate significantly higher than that of the thinner, lateral endoderm of the same level.

In all of the *somite stage* embryos the mitotic rate was higher in the intermediate endoderm than in the lateral endoderm at each level. The difference was significant in level 2 (2 specimens) and level 3 (3 specimens). Thus, in each of the somite stage embryos (and in one of the head fold stage ones) part of the endoderm which was becoming thickened as the floor or presumptive floor was proliferating more rapidly than the thinner endoderm in the neighbouring extra-embryonic area pellucida. Moreover, in 3 of these 5 specimens which had a high mitotic rate in the floor or presumptive floor it was found that the cells in the posterior part of the floor endoderm divided at a significantly lower rate than did those in one or other of the more anterior parts.

Another feature of the somite stage specimens was that the mitotic rate in the central endoderm at each level was often less than that in the intermediate endoderm at the same level (although only significantly so in level 2 of specimen 10), but greater than that in the lateral endoderm at the same level (see Table 2).

No significant differences between the mitotic rate in different parts of the extra-embryonic area pellucida endoderm were found in any of the head process or head fold stage specimens.

DISCUSSION

The validity of any deductions made about the role of mitosis during an embryonic process are first of all dependent upon the accuracy of the counting. Individual interpretation of the criteria by which the various mitotic phases are recognized is well known to affect the results of even the most careful observer. The earliest stages of prophase, for instance, are notoriously difficult to recognize. If fairly regular proportions of the phases are obtained by any investigator, however, small differences between his results and those of others in the actual mitotic rate for different regions and stages are immaterial. It is the trends which are of more significance and these will show up despite individual errors.

The results obtained for the endoderm as a whole by Schultz (1922) and Derrick (1937) are of the same order as the ones presented in this paper. Schultz gives the following average figures: 8–20 hours, 2·494 per cent.; 33 hours, 1·952 per cent.; 48 hours, 2·611 per cent. The figures given by Derrick, whilst somewhat higher than the present ones, especially in the head fold stage, show a similar trend (h.p., 2·53 per cent.; h.f., 3·2 per cent.; 5-somite stage, 2·0 per cent.; 8-somite stage, 1·8 per cent.). She also gives a map for the endoderm in a primitive streak stage specimen, showing that generally the highest rates appear to be in the midline along the primitive streak and anterior to it, although she also reports some very low rates in this region. In the immediately subsequent stage, the head process one, I have also obtained in one specimen highest mitotic rates in the anterior part of the central strip, a comparable region.

It is unlikely that throughout development one chick embryo will possess a mitotic rate more than twice that of another; for in that case it would be much greater in size than the other on hatching. The large differences discovered in the present investigation between the mitotic rate in the endoderm in embryos at the same stage of development therefore suggests that the rate of division fluctuates, at least in the stages examined. The frequency of cell division also varies within each embryo from one part of the endoderm to another at any given time. The high mitotic rate found in the intermediate strip in the later stages cannot, however, be attributed to random variation since it occurred in all the somite stage embryos and in the head fold specimens. It was not apparent in any of the head process blastoderms. It was limited to the region which was thickened, becoming thickened, or destined to become thickened. Since the peak of mitotic rate was sometimes found in the actual foregut floor (e.g. specimen 10), and sometimes in the presumptive foregut floor (e.g. specimen 8), it appears that the proliferation is not concerned solely, if at all, with ventral closure of the foregut, for in that case it would be expected to be found invariably in region 3 (Text-fig. 1, B, C, and D). It is possible that the high mitotic rate may be connected with the histological differentiation taking place in that region; the change in this epithelium from flattened to cubical to columnar continues from the head fold stage until after the foregut floor has formed, each cell becoming taller and also narrower. If the width of the region is to be even maintained therefore, the number of cells must increase. Thus this thickening region would be expected to have a mitotic rate which was higher than that in the thinner regions of the endoderm which did not undergo this change. Further evidence in support of this hypothesis may be derived from a study of the effects of certain mitotic inhibitors on developing embryos; e.g. folic acid antagonists, which inhibit mitosis, frequently cause anomalies in the histological structure of the thickening region of the presumptive foregut floor (Bellairs, 1954) and the lateral extent of the thickened part is less than usual.

In the most posterior end of the floor endoderm these histological changes are delayed until a later stage when the hind gut begins to develop. This would account for the significantly lower mitotic rate found in this part of the endoderm in three of the specimens.

Except at its anterior end the roof of the developing foregut has thinner walls than any other part of the area pellucida endoderm. This might be expected to be the result of a low mitotic rate in association with the expansion of the blastoderm. Except in specimen 10 the mitotic rate of the central endoderm was, however, never found to differ significantly from that of other regions. The thinning does not therefore appear to be dependent on a low mitotic rate. Discussion of it will be postponed till a subsequent paper.

The high rate of mitotic division in level 2 of the roof endoderm of specimens 8 and 9 (Table 2) is probably due in part to the fact that the anterior tip of the foregut roof is normally thickened like that of the floor, and may thus undergo similar changes. The high rate in level 2 of specimen 2 may be due to the same reason, although it is strange that level 3 should also be affected. It is likely that a proliferating centre may exist at the tip of the roof in the other embryos but that this is not shown in the results since the relative lengths of thickness and thinness of roof present in level 2 differ in each case.

The fact that the roof endoderm did not usually differ significantly in its rate of mitosis from the lateral endoderm emphasizes the reality of the differences in mitotic rate between the floor and lateral endoderm.

SUMMARY

1. Mitotic counts have been made in the area pellucida endoderm of 10 chick embryos.

2. In the head process stage embryos there was no evidence that any region of the endoderm consistently possessed a mitotic rate which was statistically significantly different from that of any other.

3. In all the early somite stage embryos a region possessing a high mitotic rate was found in either the foregut floor or the presumptive foregut floor. It is suggested that as the histological structure of the developing foregut floor of the embryo changes from cubical to columnar, and the individual cells become

narrower, a proliferation is needed to maintain the width of the region. There is no evidence that this high mitotic rate is necessary for the morphogenetic movements leading to the ventral closure of the foregut.

4. There is no evidence to suggest that a lowering of the mitotic rate plays any part in the thinning of the roof of the developing foregut.

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REFERENCES

Bellairs, Ruth (1953*a*). Studies on the development of the foregut in the chick. I. The presumptive foregut area. *J. Embryol. exp. Morph.* **1,** 115–24.

—— (1953*b*). Studies on the development of the foregut in the chick. II. The morphogenetic movements. *J. Embryol. exp. Morph.* **1,** 369–85.

—— (1954). The effects of folic acid antagonists on embryonic development. Ciba Symposium on *The Chemistry and Biology of Pteridines*, 356–65. London: Churchill.

Derrick, G. Ethel (1937). An analysis of the early development of the chick by means of the mitotic index. *J. Morph.* **61,** 257–84.

Schultz, Audrey Flitch (1922). The mitotic index of the chick. *Proc. Oklahoma Acad. Sci.* **52,** 45–48.

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Dissociation and Reaggregation of Embryonic cells of *Triturus alpestris*

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WITH ONE PLATE

INTRODUCTION

CELL movement and migration seem to play an important role in the formation of tissue-patterns during embryogenesis. Phenomena such as the appearance of loosely attached cells in the mesoderm, or of the freely migrating neural crest cells, are quite common in embryonic development. Since the tissues of adult organs are mostly rather compact in structure it seems that the capacity of isolated or loosely arranged cells to reassociate is an obligatory condition for many developmental processes. This capacity, under experimental conditions, was extensively studied by Holtfreter (1947, 1948). He has shown that cells of newt blastulae and early gastrulae can be made to dissociate and can then become reaggregated and proceed with their morphogenetic development. His experiments were carried out, for the most part, either with cells prior to histogenetic determination or with determined cells of a single tissue. Therefore no definite conclusion could be drawn from these observations as to whether in a reaggregate of cells from heterologous tissues the cells from each tissue will become reintegrated with their like and will differentiate according to their original presumptive fate, or whether regulatory modifications of differentiation occur in the aggregate. Moscona & Moscona (1952) described the formation of histological patterns from suspensions of cells obtained from multi-tissued organ rudiments of chick embryo. The experiment reported here is an attempt to investigate this question using isolated cells from newt neurulae, i.e. cells of tissues already determined and in which histogenesis has already started.

MATERIAL AND METHODS

The animals used were embryos of *Triturus alpestris*. The tissues were neural tissue, axial mesoderm, and endoderm of Harrison's stages Nos. 14–16. The

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embryos were dissected in standard salt solution (Holtfreter's). An attempt was made at first to follow Holtfreter's method of cell-dissociation, and pieces of tissues were transferred to alkaline solutions, with a pH range from 9 to 10. It was found that these media tend to cause a rapid disintegration of the isolated cells from these stages. When a piece of tissue is placed in the solution, the peripheral cell layer gradually dissociates, but these cells undergo cytolysis before the inner cells are isolated. The content of the cytolysed cells forms a sticky mass, probably consisting mostly of denatured proteins, in which the rest of the tissue is entangled, so that no real suspension of cells can be obtained.

In view of the assumed existence of proteins in the intercellular ground substance of embryonic tissues, we have tried to follow Moscona (1952) in applying proteolytic enzymes for the dissociation of cells. 1·5 per cent. Trypsin in normal Holtfreter solution, buffered to $pH = 7{\cdot}5$, was found to cause a complete dissociation of neural tissue cells in 3–4 hours. But these cells, although viable on culturing in normal Holtfreter, could not reaggregate. Similar experiments were carried out with papain. To 100 c.c. solution of 2 per cent. papain in 10 per cent. Holtfreter, 30 mg. of Cysteine-HCl was added, and the pH adjusted to 6·6–6·8 by 1 N NaOH (Spiegel, 1951). Neural and mesodermal tissues become dissociated in 6–7 hours. But here, again, no aggregation could be obtained after the transfer of the cells to salt solutions. Similar results were obtained with 3 per cent. pepsin.

Finally, it was found that a solution of 0·017 M sodium citrate (or 0·017 M potassium oxalate) and 0·02 M glycine in Ca-free Holtfreter solution will cause a complete dissociation of the tissues, and permit their reaggregation. Pieces of tissues were placed in Petri dishes containing the citrate-glycine solution; and after about 1 hour they disaggregated. The cells were then transferred with a fine pipette to an agar-bottomed Petri dish, containing normal Holtfreter solution, the pH of which was adjusted to 6·6 by Sørensen's phosphate buffer. The isolated cells were placed in a small depression on the agar surface; reaggregation took place after 10–15 minutes. The aggregates were left in this solution for 18–20 hours, after which they were implanted into explants of ectoderm of young gastrulae (stage 10), and transferred to Holtfreter solution with 0·1 per cent. sulphadiazine (but without phosphate buffer). In this solution the explants were cultivated for about 6–7 days.

OBSERVATIONS AND DISCUSSION

The cytological transformations of neurula cells during and after dissociation in citrate or oxalate media are similar, in general, to those described for younger embryos (Holtfreter, 1948). The cells show two distinct regions of ectoplasm and endoplasm, with frequent breaks in the plasmagel wall (Plate, fig. A). These cells were transferred immediately after isolation to salt solution ($pH = 6{\cdot}6$). Here the change from a collection of completely isolated cells to a firm aggregate is practically instantaneous. It seems therefore likely that the main factor in the process of cellular adhesion is the formation of bonds between the outer components of the cell membranes rather than a gradual synthesis of an intercellular ground substance.

The first combination of cell suspensions used in these experiments was one of neural and mesodermal cells. Two days after implantation into flaps of gastrula ectoderm it was found that a short rod of cells was usually formed within the aggregate. In cross-section the 'rod' appears to possess a centre to which its cells are attached. This centre in most cases consists of cytolysing cells with pycnotic nuclei, around which the 'attached' cells are organized radially (Plate, fig. B). In later stages a lumen appears in this centre, surrounded by an epithelium in the shape of a neural tube (Plate, figs. C and D). Within this pseudo-neural canal remnants of the cytolysing cells may remain up to the 5th day (Plate, fig. C). Attempts to use vital stains in order to mark cells of different tissues have failed. Therefore it was attempted to use cells of distinct size differences. A suspension consisting mainly of small neural and large endodermal cells was allowed to reaggregate. After 5 days it could be seen that an epithelial tube was formed from the neural cells, whereas the larger endodermal cells reorganized a tube similar to the archenteron (Plate, fig. E).

None of the aggregates revealed any clear differentiation of the myogenic mesodermal cells, neither did they integrate into somite-like structures. Moscona & Moscona (1952) have pointed out that in their cultures of cell suspensions from chick limb buds the myoblasts did not differentiate typically while the chondrogenic cells reintegrated and formed definite skeletal parts.

Holtfreter (1948) stressed the importance of selective adhesiveness in aggregation of cells of different histogenetic nature. However, our observations on the behaviour of small populations of cells, in which a few mesodermal cells were put together with a few neural ones, indicate that at least during the process of aggregation cells of either tissue are fully capable of sticking to cells of the other. It seems, therefore, that, at least as far as amphibian embryonic cells are concerned, reaggregation and the organization of histotypic patterns within the aggregates are distinct processes. Any regrouping which does take place within the aggregate is apparently a result of migration which starts after aggregation. Nothing can yet be said about the factors determining the direction of this migration. Whether the appearance of cytolysing cells in the centre of the reformed 'neural tube' is of any significance to this process remains a matter for further study.

There is as yet no evidence for the existence of a cytotropic mechanism in the attraction of embryonic cells, suggested by Roux (1894). Holtfreter (1948) assumes that the meeting of particular cells within the aggregate is a matter of chance, but that physico-chemical properties of their surfaces determine whether or not final adhesion between the cells will take place. According to this view, the behaviour of embryonic cells of newts is not the same as that of Myxomycetes, in which chemical cytotaxis in the formation of the plasmodium has been described (Bonner, 1947).

Attempts to culture the aggregates without implanting them into ectodermal explants resulted in their complete dissociation and cytolysis. The main purpose of the implantation was to supply the cells with a 'surface-coat'; the importance of this for the integrity of the newt embryo is well known.

The failure to get aggregation from cells isolated by means of proteolytic enzymes is in accordance with observations on the effects of trypsin on the surface coat of younger embryos (Townes, 1953). Cytological differences between cells dissociated by citrate and those dissociated by enzymes, such as the formation of huge pseudopodia by the latter, suggest that the protein components of the cell membrane may be affected by proteolytic enzymes. It was pointed out recently that chelating agents, like versene, may cause rabbit blastomeres of the two-cell stage to dissociate (Brochart, 1954). In other biological systems, glycine has been shown to act like versene in its chelating function, but with less toxic effects (Tyler, 1953). It is, therefore, possible that in the process of cell dissociation glycine acts as a chelating agent, parallel with the action of the citrate and oxalate on the Ca-ions of the cell membranes.

SUMMARY

1. The dissociation of cells of neurulae of Triturus by means of sodium citrate or potassium oxalate and glycine is described.

2. Suspensions of isolated cells of neural, mesodermal, and endodermal tissues were made to reaggregate in a slightly acid solution, and the aggregates were cultured within explants of ectoderm.

3. Reintegration of some histogenetic patterns was observed. Differentiation of structures resembling neural tube and archenteron are described in aggregates of neural and endodermal tissues.

4. Some factors involved in the dissociation and reaggregation processes are briefly discussed.

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REFERENCES

Bonner, J. T. (1947). Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum. *J. exp. Zool.* **106,** 1–26.

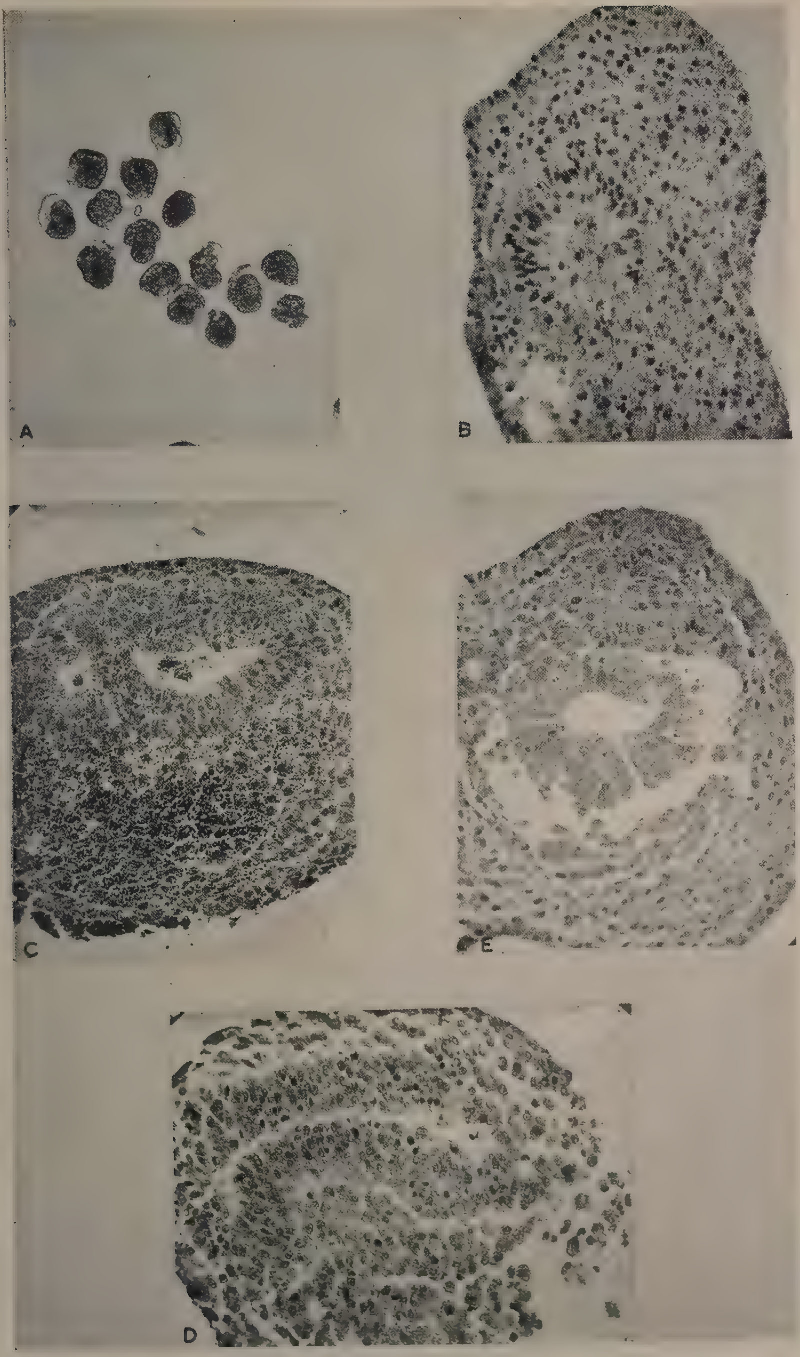
Brochart, M. (1954). Attempted experimental production of identical twins in rabbits. *Nature, Lond.* **173,** 160–2.

Holtfreter, J. (1947). Observations on the migration, aggregation, and phagocytosis of embryonic cells. *J. Morph.* **80,** 25–55.

—— (1948). Significance of the cell membrane in embryonic processes. *Ann. N.Y. Acad. Sci.* **49,** 709–68.

Moscona, A. (1952). Cell suspensions from organ rudiments of chick embryos. *Exp. Cell Res.* **3,** 535–9.

—— & Moscona, H. (1952). The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J. Anat., Lond.* **83,** 287–301.



M. FELDMAN

Plate 1

Roux, W. (1894). Über den 'Cytotropismus' der Furchungszellen des Grasfrosches (Rana fusca). *Arch. EntwMech. Org.* **1,** 43–68.

Spiegel, M. (1951). A method for the removal of the jelly and vitelline membrane of the egg of Rana pipiens. *Anat. Rec.* **111,** 544.

Townes, P. L. (1953). Effects of proteolytic enzymes on the fertilization membrane and jelly layers of the amphibian embryo. *Exp. Cell Res.* **4,** 96–101.

Tyler, A. (1953). Prolongation of life span of sea urchin spermatozoa and improvement of the fertilization reaction by treatment of spermatozoa and eggs with meto-chelating agents (amino-acids, versene, DEDTC, oxene, cupron). *Biol. Bull., Wood's Hole,* **104,** 224–50.

EXPLANATION OF PLATE

Fig. A. Dissociated cells of neural tissue (stage 14). ×211.

Fig. B. An aggregate, two days after implantation. Neural cells are organized radially around a centre of cytolytic cells. ×125.

Fig. C. An aggregate, 5 days after implantation. In the central canal of the reformed 'neural-tube' are remnants of cytolysing cells. ×130.

Fig. D. Longitudinal section through a 'neural-tube' in an aggregate, 6 days after implantation. ×130.

Fig. E. An aggregate of neural and endodermal cells, 5 days after implantation. The neural cells reorganized a 'neural-tube', while the endodermal ones formed a larger tube, similar to the archenteron. ×125.

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Post-Partum Changes in the Uterus of the Rat

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WITH TWO PLATES

INTRODUCTION

THE changes that occur in the uterus of the rat after parturition have been described by Duval (1891) and by Strahl (1906). In this animal, at the time of parturition, the uterine mucosa is lined everywhere by epithelium except at the placental sites. Duval expressed the view that the area of endometrium not covered by epithelium after the separation of the placenta was re-epithelialized by a transformation of endometrial stromal cells into epithelial cells, and he claimed to have observed cells in the various stages of this process. On the other hand, Strahl, who worked with the mouse, was of the opinion that the bare area was recovered by a spread of cells from the epithelium at its margin. He considered that this also occurred in the rat and in other mammalian species. Bull (1949), studying the rabbit, was unable, on the material available to him, to confirm either of these views. Sharman (1951) investigated the effect of ovariectomy and the administration of oestradiol monobenzoate and progesterone on the uterus of the post-partum rat, but he makes no comment on the method of regeneration of the epithelium at the placental site.

This investigation was undertaken in order to study: (1) The mode of regeneration of the epithelium at the placental site in the rat; (2) the time at which regeneration is completed; and (3) the distribution of glycogen and ribonucleic acid in the regenerating epithelium. In addition, certain observations were made on some other changes that occur in the uterine epithelium and on the behaviour of the endometrial stroma.

MATERIALS AND METHODS

To ensure standard conditions, particularly with regard to the hormonal state, this work was carried out on lactating animals with six or more offspring, and with the possibility of further pregnancy excluded.

Virgin rats were mated and used for observation provided they had a litter of six or more and were lactating. After littering the females were segregated from

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the males. Seven animals were killed immediately after parturition was completed (referred to as 0 hours). Five animals were sacrificed 6 hours after parturition, three at 12 and 18 hours, two at 24 and 30 hours, and three at 36 hours. Then five animals were killed on the 2nd day, three on the 3rd day, two on the 4th day, and four on the 5th day. Portions of the uterine horns containing placental sites were removed and fixed in either Bouin's fluid or ice-cold Rossman's fluid (9 parts of absolute alcohol saturated with picric acid, and 1 part of neutral formalin). It was possible to recognize the position of the placental sites without opening the uterine horns, partly because the horns are thicker in these regions and partly by means of the metrial gland which bulges into the mesometrium at each site. After embedding in paraffin wax, serial sections were cut and mounted. Those that had been fixed in Bouin's fluid were stained with haemotoxylin and eosin. The material fixed in Rossman's fluid was treated to show the presence of either glycogen or of ribonucleic acid. For the demonstration of glycogen the periodic acid-Schiff (PAS) reaction was used. Control sections from which glycogen had been eliminated by digestion with diastase (Gomori, 1952) prior to staining were also prepared.

To show cytoplasmic basophilia sections were stained for 15 minutes in a 0·2 per cent. aqueous solution of toluidine blue and dehydrated in butyl alcohol for 10 minutes before clearing and mounting. Cytoplasmic basophilia, due to ribonucleic acid, was recognized by incubating control sections in 10 per cent. perchloric acid for 12 hours at 4° C. before staining—a procedure which extracts ribonucleic acid from the section.

In a number of animals one uterine horn was opened and the placental sites examined with the naked eye.

OBSERVATIONS

The uterus at 0 hours

Although the uterus collapses immediately after littering and is reduced in size by contraction of its muscle coat, it is still much larger than before pregnancy. Measurements made on transverse sections taken through the placental site showed the uterine horn to be about 4·25 mm. in width. On opening a uterine horn, the placental sites can be recognized along the mesometrial border of the mucosa. They are about 1 to 2 mm. in diameter and show as deep pits, usually filled with blood clot.

The uterine mucosa is thrown into folds which vary in size and shape and run in the long axis of the horns. In the region of the placental sites one or more large folds project into the uterine lumen, incompletely dividing the latter into a larger antimesometrial portion and a smaller mesometrial one (Plate 1, fig. A). Duval referred to these as the utero-placental folds.

The mucosa, except at the placental sites, is lined by a single layer of tall, non-ciliated, columnar cells (Plate 1, fig. B), which tend to become cuboidal at the edges of the folds. The cell outlines are distinct and the cytoplasm contains fine granules especially in the supranuclear region. At the free surface of the cell there is a clear eosinophilic layer referred to by Pritchard (1949) as the distal band. The nuclei which are situated towards the bases of the cells are round to oval in shape, vesicular in appearance, and contain one or two nucleoli. When stained with toluidine blue the cells show marked cytoplasmic basophilia, particularly in the supranuclear region (Plate 1, fig. B). In the control sections there is a marked reduction in cytoplasmic basophilia and it is, therefore, concluded that the original basophilia was due to ribonucleic acid (Plate 1, fig. C). With the PAS technique the cells show a number of small granules, mainly in the supranuclear region, a surface layer of PAS positive material, and a distinct basement membrane (Plate 1, fig. D). The appearance is similar in the control sections and is, therefore, not due to glycogen. This distribution of PAS positive material corresponds to that described by Leblond (1950) in the epithelium of the non-pregnant rat uterus.

Mitotic figures are numerous throughout the epithelium except in regions adjacent to placental sites where there are only a few. In the non-placental regions as many as forty mitoses can be seen in a single tranverse section.

At intervals between the typical columnar cells a second type of cell can be distinguished. These are narrow and elongated with deeply staining cytoplasm and spindle shaped nuclei. They resemble the rod cells described by Horning (1942–4) in the uterine epithelium of the non-pregnant rat, and by Aykroyd & Gatenby (1941) in man. Mitotic figures can occasionally be seen in these cells.

The epithelium terminates abruptly at the site of insertion of the placental pedicle. At this stage the epithelial cells at the margin of the bare area are cuboidal in shape. The epithelium near the placental site is infiltrated by a large number of polymorphonuclear leucocytes.

Glands, in small numbers, can be seen in the side walls of the uterine horn and to a lesser extent along the antimesometrial border but they are absent from the mesometrial border and thus from the placental sites.

The endometrial stroma is most abundant in the folds. Between the folds the epithelium is always separated from the myometrium by a thin layer of stroma. The cell density of the stroma varies. In the larger folds there are a few cells scattered in an abundant ground substance, giving an oedamatous appearance. Elsewhere, especially at the bases of the folds, the cells are more numerous and the ground substance reduced in amount. The stromal cells, which resemble fibroblasts, show no evidence of a decidual reaction except round the placental site where there are a few cells containing glycogen.

In the subepithelial zone the stromal cells are orientated parallel to the surface but otherwise there is no regular arrangement. The blood-vessels passing through the stroma are dilated and the subepithelial plexus is well marked.

At the placental site there is no epithelium and the stromal cells are more numerous than elsewhere. The placental artery enters the stroma through a gap in the circular layer of the myometrium, the fibres of which, in this region, are widely separated by cells which resemble those of the endometrial stroma. The artery, which passes through the stroma towards the surface of the bare area, contains a mass of thrombus and there are a large number of basophilic multinucleate giant cells within its wall. Between the giant cells the wall is for the most part made up of a homogeneous eosinophilic staining material, which colours with the PAS reaction. This material, apart from its general distribution in the vessel wall, is present in large amounts round the giant cells so that each cell appears to be enclosed in a capsule. Its presence is taken to indicate a hyaline degeneration of the vessel wall. On the surface of the bare stroma there is a mass of blood clot which may extend into the stroma on either side of the placental artery. Blood clot may be present in the stroma itself or between the stroma and the uterine epithelium. Polymorphonuclear leucocytes are already present in the blood clot and adjacent endometrium.

The uterus from 6 to 12 hours post-partum

By 12 hours post-partum the uterine horns have decreased slightly in size, being about 4 mm. in width in the placental regions, but the lumen remains large and the endometrium is still extremely folded.

The re-epithelialization of the placental site commences soon after littering. By 6 hours the epithelial cells at the margin of the bare area vary in shape (Plate 1, fig. E). In sections some appear round, others oval, pear-shaped, or flat. The nuclei are enlarged with prominent nucleoli and the cytoplasm is less basophilic, contains no glycogen, and there is no basement membrane, while the intercellular boundaries are indistinct. These hypertrophic cells have a thin layer of PAS positive material on the free surface and form a single cell layer continuous with adjacent columnar epithelial cells. In some places they lie directly on the stroma but elsewhere are separated from the stroma by blood clot or fibrin clot. There is no evidence of a transformation of stromal cells into epithelial cells. At 6 hours the hypertrophic cell layer is about 12 to 18 cells in width. Similar appearances are present at 12 hours although now the hypertrophic cells are more numerous.

The remainder of the epithelium shows little change. The cells remain tall and columnar, but at 12 hours the bases of the cells have become more eosinophilic and in the same region the intercellular boundaries are less distinct. No mitoses were found anywhere in the epithelium although several dozen sections were examined. The leucocytes' infiltration now extends throughout the whole of the epithelium. Rod cells are no longer present (Plate 2, fig. F).

The uterus from 18 to 24 hours at post-partum

The reduction in size of the uterus continues and the folding of the endometrium persists. The uterine horns in the placental regions are now about 3·5 mm. wide. The placental site is extensively re-epithelialized; in an occasional specimen, completely so. The new epithelium consists of the hypertrophic cells previously described. Plate 2, figs. G and H are of a section taken from a specimen 24 hours after littering, where the placental site is completely re-epithelialized. The new epithelium consists of a single layer of hypertrophic cells extending over a mass of blood clot and is continuous with the adjacent columnar epithelium. The covering hypertrophic cells still retain their enlarged nuclei, diminished cytoplasmic basophilia, while glycogen and the basement membrane are absent.

Further changes have occurred elsewhere in the uterine epithelium. At 24 hours the eosinophilia at the bases of the cells is more marked and here the cell outlines are unrecognizable. Many of the cells are vacuolated. The vacuoles are mainly in the supranuclear part of the cell. In size they are as large or larger than the nucleus and many of them contain debris (Plate 2, fig. I). The extent of the vacuolation varies in different regions of the epithelium. In some places the cells have a normal appearance while in adjacent regions vacuolation may be extensive and in such regions it is difficult to identify the boundary between epithelium and endometrial stroma, although with PAS material a basement membrane can always be seen. The cells remain tall, but in places they appear pseudostratified. Mitoses were not found.

The density of the cell population of the stroma has increased but remains relatively greater in the region of the placental sites. The blood-vessels continue to be dilated, while organization of the placental artery has progressed.

The uterus from 30 to 36 hours post-partum

The uterus has further decreased in size, and by 36 hours the horns are about 3 mm. wide in the placental regions. The folding of the endometrium persists but the folds are fewer and larger. In all the specimens studied the placental site bare area is completely re-epithelialized by 36 hours although the covering cells may still be oval or pear-shaped.

In the rest of the epithelium the vacuolation is now more marked. More cells show vacuoles containing debris, but there are patches of normal epithelium (Plate 2, fig. J). Where there is marked vacuolation the boundary between epithelium and stroma is indefinite in specimens stained with haemotoxylin and eosine, although the basement membrane is visible in PAS stained sections. No mitoses were visible. Leucocytic infiltration continues.

The stroma is more abundant and more cellular but the greater density of the cells in the placental regions persists. The stromal vessels are less prominent.

The uterus at 48 hours post-partum

The uterus has decreased considerably in size, and in the regions of placental sites the horns are about 2·25 mm. in width. The endometrium is no longer folded and the lumen is present as a narrow slit which runs from the mesometrial to the antimesometrial border. At the placental site the epithelial cells are similar to those found elsewhere in the uterus. There is no vacuolation and the cells are low columnar in shape with central nuclei (Plate 2, fig. K). A few mitotic figures are present. Rod cells have reappeared. Only an occasional polymorphonuclear leucocyte can be found in the epithelium.

The stroma forms a thick cellular layer separating the epithelium from the myometrium. The cell density remains greatest in the placental regions. The part of the placental artery that lies within the endometrium shows marked organization. Some of the giant cells have become separated from the wall of the artery and lie in the endometrial stroma. They appear healthy and retain their covering of eosinophilic PAS positive material. Along the course of the artery there are groups of cells which contain small brown pigment granules. These granules stain with the PAS reaction. The dilatation of the stromal vessels has subsided.

The uterus from 3 to 5 days

There are few additional changes during this period. By 5 days the stromal cell population at the placental site is the same as elsewhere. In some cases the remains of the placental artery have completely disappeared from the stroma while in others only a small fragment of the wall remains. There are more pigment containing cells present and the pigment granules which are still PAS positive are larger. These cells tend to remain near the sites grouped in the endometrium adjacent to the circular muscle coat, and in the metrial glands. A few giant cells are present either in the endometrium or the metrial gland. Although breakdown of the placental artery is nearly complete within the endometrium it still continues within the metrial gland.

DISCUSSION

In the material examined the placental site clearly seemed to be epithelialized by a spread of cells from the surrounding uterine epithelium. At no time were there any isolated patches of regenerating epithelium, and at all stages this new epithelium of the placental site was continuous with the original epithelium. The hypertrophy, varied form of the cells, and absence of mitotic figures are characteristics of spreading epithelium. For example, they are evident in the epithelium spreading in to repair corneal or skin defects (Arey, 1936). Moreover, in many sections the epithelium was resting on blood clot or fibrin clot and not directly on the stroma. Where the regenerating epithelium was lying directly on the stroma there was an abrupt demarcation between epithelial and stromal cells. There was no evidence of any transformation of stromal cells into epithelial cells. As there were no glands at the placental site there was no question of regeneration from glandular epithelium. Glycogen was absent from the normal and regenerating uterine epithelium, which, in this respect, differs from regenerating skin epithelium with its abundant glycogen (Bradfield, 1951), especially in the superficial layer of the stratum spinosum. This is probably related to the later keratinization of skin which, of course, does not occur in the uterine epithelium. Ribonucleic acid, although present in the normal uterine epithelial cell, was diminished or absent from the regenerating epithelium which, in this respect, is similar to regenerating skin epithelium.

The relatively small bare areas of the placental sites were in this series completely re-epithelialized within 36 hours after littering. This rapid regeneration is probably related to the early post-partum oestrus; in the rat the first post-partum ovulation occurs about 18 hours after littering.

During involution, areas of the uterine epithelium show vacuolation of the columnar cells which is particularly marked 36 hours post-partum. Long & Evans (1922) described a similar vacuolation of the epithelial cells during oestrus which they referred to as vacuolar degeneration. The post-partum vacuolation may be related to the onset of the first post-partum oestrus, but may also signify a degeneration of epithelial cells consequent upon the decrease in size of the involuting uterus.

Hamilton (1933) found that in the guinea-pig the uterine epithelium is shed about the 3rd day post-partum and is replaced from the epithelium of the uterine glands. A similar process, occurring on the 1st day post-partum, has been described by Bull (1949) in the rabbit, but a comparable shedding and replacement of the epithelium were not seen in the rat.

In man, according to Williams (1931), the placental area is cleared by a process which he calls exfoliation. This is characterized by the undermining of thrombosed vessels and tissue debris by the ingrowth of new endometrium, with subsequent shedding of the necrotic material into the uterine lumen. According to Dano (1937) exfoliation also occurs in the placental sites in the mouse. However, no evidence of exfoliation was found in the rat. It would thus appear that the pattern of post-partum repair varies considerably in different species. This may be in part due to differences in the details of placental development and in part related to the time of occurrence of the first post-partum ovulation.

SUMMARY

1. Rats were killed at intervals from 0 hours to 5 days after littering. Some of the changes that occur in the uterus during this period were studied.

2. The area of endometrium left devoid of epithelium by the separation of the placenta was re-epithelialized by cells which spread in from the existing marginal epithelium. There was no evidence of a transformation of stromal into epithelial cells.

3. The migrating epithelial cells did not contain glycogen and their cytoplasmic content of ribonucleic acid was reduced.

4. Re-epithelialization of the sites was completed within 36 hours.

5. There was vacuolation of the epithelial cells which was most marked from between 24 to 36 hours after littering.

6. The changes in the rats' uterus are compared with those occurring in some other mammals.

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REFERENCES

AREY, L. B. (1936). Wound healing. *Physiol. Rev.* **16,** 327–406.

AYKROYD, O. E., & GATENBY, J. B. (1941). Cytology of human uterine glands in gravid and non-gravid phases. *Quart. J. micr. Sci.* **82,** 541–62.

BRADFIELD, J. R. G. (1951). Glycogen in vertebrate epidermis. *Nature, Lond.* **167,** 40–41.

BULL, M. M. (1949). Repair of the placental site in the rabbit. *J. Obstet. Gynaec.* **56,** 799–818.

DANO, R. A. (1937). Uterine macrophages in the mouse and their relation to involution. *Amer. J. Anat.* **60,** 433–71.

DUVAL, M. (1891). Le Placenta des Rongeurs. *J. Anat., Paris,* **27,** 515–612.

GOMORI, G. (1952). *Microscopic Histochemistry.* Chicago: The University of Chicago Press.

HAMILTON, W. J. (1933). The restoration and regeneration of the epithelium and endometrium of the uterus of cavia post partum in non-pregnant animals. *Trans. roy. Soc. Edin.* **57,** 593–600.

HORNING, E. S. (1942–4). The reaction of the uterine epithelium of the rat to oestrogenic stimulation. *J. Endocrinol.* **3,** 260–7.

LEBLOND, C. P. (1950). Distribution of periodic acid-reactive carbohydrates in the adult rat. *Amer. J. Anat.* **86,** 1–50.

LONG, J. A., & EVANS, H. M. (1922). The oestrus cycle in the rat and its associated phenomena. *Mem. Univ. Calif.* **6,** 1–148.

PRITCHARD, J. J. (1949). Alkaline phosphatase in the uterine epithelium of the rat. *J. Anat., Lond.* **83,** 10–24.

SHARMAN, A. (1951). An experimental study of post partum endometrial repair in the guinea pig and rat. *J. Endocrinol.* **8,** 162–8.

STRAHL, H. (1906). Vom Uterus Post Partum. *Ergebn. Anat. EntwGesch.* **15,** 581–627.

WILLIAMS, J. W. (1931). Disappearance of the placental site during the puerperium. *J. Amer. med. Ass.* **97,** 523–9.

EXPLANATION OF PLATES

PLATE 1

FIG. A. Transverse section of uterus immediately after littering. Note blood clot at the placental site and the folding of the mucosa. Stained with haemotoxylin and eosin. ×6·5.

FIG. B. Part of a transverse section of uterus at 0 hours post-partum stained with toluidine blue. Note the intense staining of the cytoplasm of the epithelial cells. ×485.

FIG. C. Portion of a transverse section of uterus from the same animal as Fig. B, stained with toluidine blue after extraction with perchloric acid. Note the decrease of cytoplasmic basophilia. ×485.

FIG. D. Transverse section of uterus from the same animal as Fig. B, stained by the PAS technique and showing cytoplasmic granules, surface PAS positive layer, and basement membrane. ×485.

FIG. E. Part of a transverse section of uterus 6 hours after littering, showing the edge of a placental site. Stained haemotoxylin and eosin. ×105.

PLATE 2

FIG. F. Transverse section of uterus 12 hours post-partum. Note the infiltration of polymorphonuclear leucocytes in the epithelium. Stained haemotoxylin and eosin. ×485.

FIG. G. Transverse section of a placental site 24 hours post-partum. The site has been re-epithelialized and the hypertrophic epithelial cells are resting on a mass of fibrin clot. Stained toluidine blue. ×105.

FIG. H. Part of Fig. E at a higher magnification. Normal epithelium to the left and hypertrophic cells to the right. Note the reduced cytoplasmic basophilia of the hypertrophic cells. ×485.

FIG. I. Transverse section of uterus from same animal as Fig. E. Epithelial cells show early vacuolation. Haemotoxylin and eosin. ×485.

FIG. J. Transverse section of uterus 36 hours post-partum showing extensive vacuolation of epithelial cells. Haemotoxylin and eosin. ×485.

FIG. K. Transverse section of uterus 48 hours post-partum. Note the low columnar epithelium and absence of vacuolation. Haemotoxylin and eosin. ×485.

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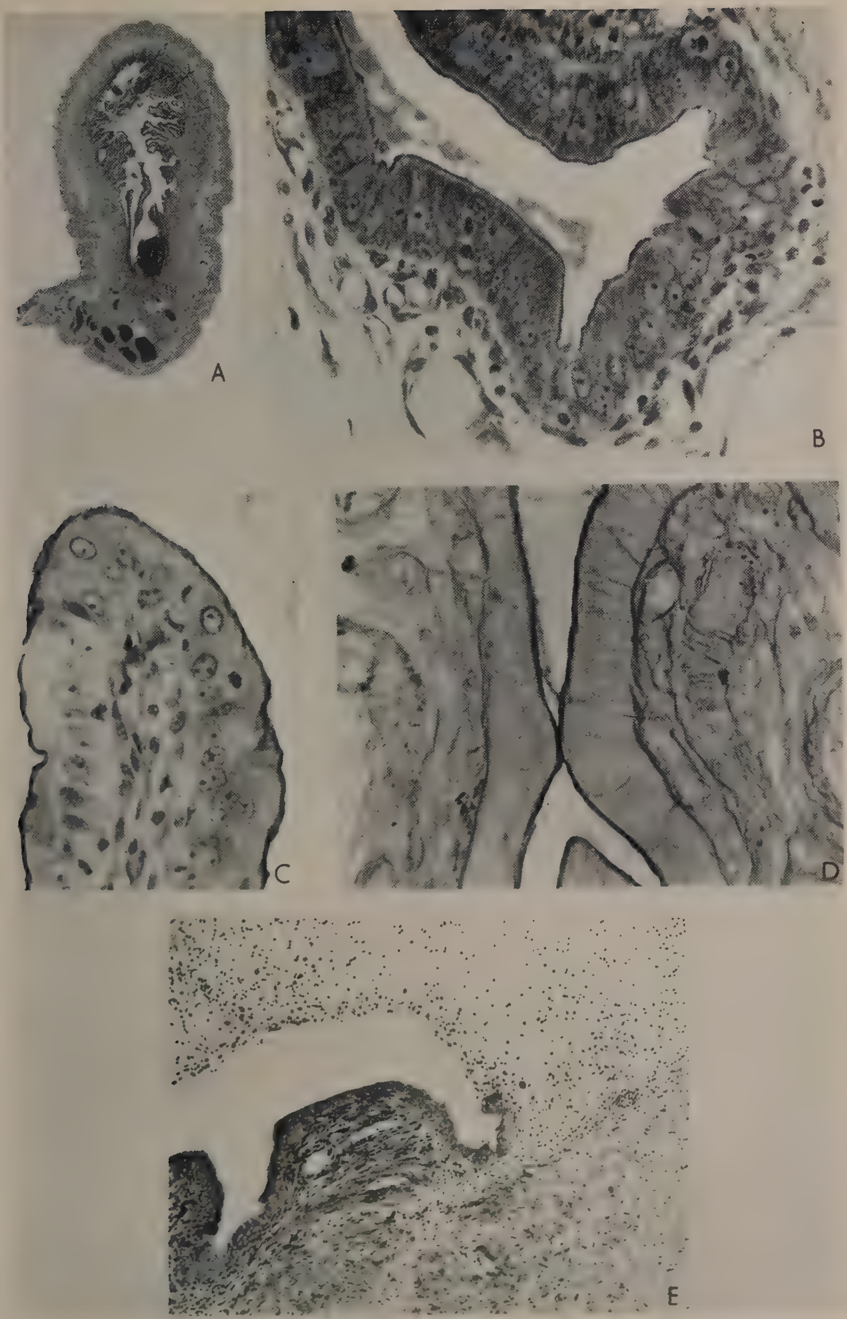
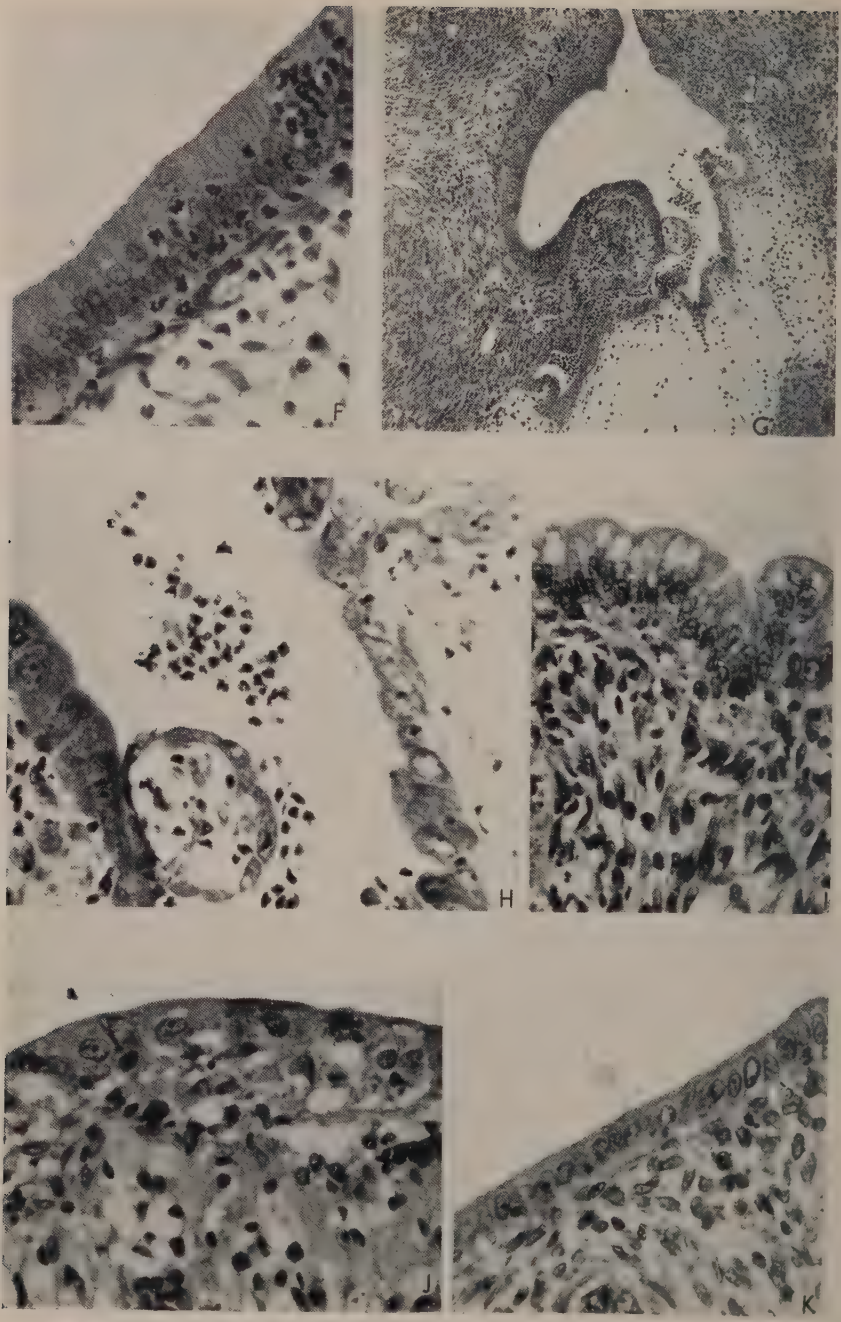


J. G. WARBRICK

Plate 1



J. G. WARBRICK

Plate 2

The Effect of Prior Intravenous Injections of Dissociated Epidermal Cells and Blood on the Survival of Skin Homografts in Rabbits

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WITH TWO PLATES

INTRODUCTION

THE epidermal component of a skin homograft has recently been shown to be almost as effective in eliciting transplantation immunity as the anatomically intact skin from which it was prepared (Billingham & Sparrow, 1954; Amos, Gorer, Mikulska, Billingham, & Sparrow, 1954). The primary object of the work to be described in the present paper was to determine whether prior intravenous injections of suspensions of dissociated homologous epidermal cells affect the recipient's subsequent response to skin homografts from the donor of the epidermal cells. It may be recalled that Medawar (1946) has shown that in rabbits the immunizing effect of homologous leucocytes towards subsequent skin homografts from the same donor is 18 times more effective through the intradermal route than when introduced intravenously.

MATERIAL AND METHODS

The subjects of these experiments were adult rabbits, weighing between 1·7 and 3·7 kg., from dealers' mixed stocks. The majority of these animals were males, though a few non-pregnant females (see Heslop, Krohn, & Sparrow, 1954) were also used. Donors and their recipients were deliberately selected from different breeds whenever possible in order that the genetic disparity between them should be maximal. This is the theoretical requirement for uniformity of response from different recipients to skin homografts from different donors. For the majority of experiments paired donors and recipients have been employed, though in some, two or more recipients shared a donor in common.

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1. *Epidermal cell suspensions*

The epidermal cell suspensions were prepared from very thin shavings of skin of even thickness—the thinnest possible 'split-thickness' or Thiersch grafts of the plastic surgeon—cut from the dorsa of the ears. The epidermis was first disengaged from the dermis by an enzymic digestion process and its exposed Malpighian layer covered with a thin film of normal saline containing 0·8 per cent. sodium citrate. By lightly scraping the raw surfaces of these sheets of epidermis all the basal layer cells were removed, leaving the fairly robust cuticle behind. The detached cells were taken up in 0·8 per cent. citrate-saline and thoroughly agitated with a fine pipette to break up the large cell aggregates. Examination under the microscope confirmed that practically all the cells were completely dissociated and that the degree of contamination of the Malpighian cells with cells from the higher epidermal strata was negligible. Details of the method for preparing these cell suspensions and evidence of their viability have been given in full elsewhere (Billingham & Medawar, 1951; Billingham & Reynolds, 1952).

In the early experiments to be described it was assumed that from comparable aggregate areas of skin shavings approximately similar quantities of dissociated epidermal cells could be obtained. As far as possible, for each of these experiments a constant or standard amount of skin was therefore removed from one or, in some cases, both ears of a donor for the preparation of a cell suspension. The quantity of cells injected into any recipient could then be made a fraction or multiple of the standard. In later experiments it was found that satisfactory counts of Malpighian cells could easily be made with the aid of a standard haemocytometer and the crude and subjective method of comparing the dosages administered was abandoned thereafter. Trials have shown that the previously adopted standard amount of skin shavings yielded approximately 10–12 million dissociated cells, depending partly upon the state of physiological activity of the skin from which the shavings were cut. The dosages of cells administered to the various recipients in the early experiments have therefore been estimated retrospectively on this basis.

The skin of a rabbit's trunk, which bears very dense fur, is unsuited to the preparation of epidermal sheets, so that the upper limit of the dosages of cells we have been able to employ was set by the size of the donor's ears. The areas from which skin shavings were removed healed with such rapidity that more shavings could be cut from the same donor site within about 2 weeks to provide cells for a second injection when necessary.

The volumes of the suspensions were kept within the limits of 4–8 ml. For some experiments the cells were washed before injection. This entailed spinning down the initial suspension at fairly low speed, removing the faintly opalescent supernatant, and then re-suspending the cells in the same volume of fresh citrate-saline.

All intravenous injections were carried out very slowly through the marginal ear vein. In animals receiving a second injection this was administered within 12–14 days of the first.

2. *Assessment of the effect of the epidermal cell injections*

Although 'protective' iso-antibodies have been demonstrated in the sera of rabbits that have reacted against skin homografts (Billingham & Sparrow, 1954), there is no suitable technique available at present for the routine detection and estimation of these antibodies. Nevertheless, the existence of a state of immunity directed against homologous skin from a donor may easily be recognized by the highly modified response of skin homografts transplanted to a putatively immune recipient. Suppression of epithelial proliferation, precocity of vascular stagnation and disruption of vessel walls, and the accelerated destruction of the entire graft epithelium are the essential diagnostic features.

Twenty-two to 24 days after receiving the primary injection of cells an animal received 5 standard 'pinch' grafts of its donor's skin. Each graft was a thin circular disk about 9 mm. in diameter and comprised the full thickness of the corium and epidermis. The grafts were transplanted to an extensive rectangular bed, about 6 cm. × 7 cm. in lengths of side, prepared on the side of the chest by removing the skin down to the level of the vascular fascial layer that overlies the panniculus carnosus muscle. Since the grafts were transplanted in 'open style', each could enlarge its epithelial surface by outgrowth over the raw area separating it from its neighbours or from the host's skin at the margins (Plate 1, fig. A). The operative procedure and dressings applied have been described in full elsewhere (Billingham & Medawar, 1951).

Primary inspection of the grafts was carried out on the sixth day after transplantation and subsequent inspections were made at 3-day intervals as required.

Biopsy specimens, comprising small portions of a representative graft and any epithelium that had grown out from its margin, were taken at every inspection. After fixation in formol-mercuric chloride and embedding in paraffin wax, transverse sections were cut at 8–10μ and stained with Ehrlich's haematoxylin and eosin. From the histological examination of these specimens the survival time of the epithelium of the grafts was assessed and the type of reaction—whether 'immune' or otherwise—determined.

OBSERVATIONS AND RESULTS

1. *The survival time of skin homografts in untreated rabbits*

The panel of 16 controls published by Billingham, Krohn, & Medawar (1951), which has been greatly increased subsequently (Billingham & Medawar, 1953; Billingham & Sparrow, unpubl.), has established that skin homografts, exchanged in a dosage slightly higher than that adopted in the present study, between rabbits selected for their genetic disparity, do not survive their transplantation for longer than 9 days. Provided that precautions are taken to ensure that the homograft reaction in rabbits will be of maximal violence, it is so consistent that a survival time of 12 days is a strong indication that the reaction has been modified by experimental interference (see Billingham & Medawar, 1953).

Medawar (1944) has shown that 'second-set' homografts, i.e. those transplanted from the original donor to a rabbit that has already received and reacted against grafts of its donor's skin, do not survive for longer than 7 days, their median survival time being 6·0 ± 0·6 days.

2. *First series of experiments*

The first 2 of the 19 experiments set out in Table 1 were carried out concurrently on independent paired donors and their recipients, one of the latter being injected with about twice as many cells as the other. The outcome of grafting these two animals with their respective donors' skin 22 days later was as follows: The grafts on SR-203, which had received the higher dosage of cells, succumbed after 7 days to a perfectly normal homograft reaction, there being

TABLE 1

The survival times of skin homografts transplanted to animals previously injected intravenously with their donors' epidermal cells. (First series of experiments)

Donor	*Recipient*	*Number* of cells injected (millions)*	*Condition of cells injected*	*No. of injections*	*Survival time of skin homografts (days)*	
					First set	*Second set*
SR-202	SR-203	10–12	Unwashed	1	7	—
SR-204	SR-205	5–6	"	1	>20	14
SR-204	SR-220	2·5–3	"	1	9	—
SR-204	SR-221	1·25–1·5	"	1	13	—
SR-219	SR-222	6·5–8	"	1	9	—
SR-219	SR-223	3·5–4	"	1	⩾9†	—
SR-229	SR-230	1·25–1·5	"	1	7	—
SR-229	SR-231	10–12	"	1	7	—
SR-232	SR-233	5–6	"	1	7	—
SR-232	SR-234	2·5–3	"	1	8	—
SR-232	SR-235	1·25–1·5	"	1	8	—
SR-232	SR-236	0·65–0·75	"	1	10	—
SR-237	SR-238	10–12	Washed	1	18	—
SR-240	SR-242	12–15	"	1	8	—
SR-246	SR-248	12–15	"	1	8	—
SR-252	SR-254	12–15	"	2	>18	>16
SR-258	SR-262	10–12	"	2	11	—
SR-263	SR-264	10–12	"	2	6	—
SR-268	SR-269	12–15	"	2	8	—

* The actual number of cells injected has been estimated retrospectively; see text.

† Animal died under anaesthetic at the 9-day inspection when its grafts showed no indication of an incipient reaction.

no suggestion that the breakdown was of the 'immune' type. In SR-205, however, there was still surviving homologous epithelium present on the 20th day following transplantation, when the processes of wound contracture and ingrowth of native epithelium from the margins of the operation field were so advanced that subsequent distinction between native and homologous epithelium was impossible. Twenty-nine days after its first grafting operation this animal again received skin grafts from its donor. These grafts, too, showed a significant prolongation of survival, for breakdown was not complete until the 14th day. We had, therefore, good grounds for believing that the anomalous survival of the homografts on SR-205 was a consequence of its prior injection with epidermal cells. It seemed likely that the known disparity between the numbers of cells injected into the two recipients was responsible for the difference between the two results.

Subsequent experiments, summarized in Table 1, were carried out in the hope of reproducing this unexpected and interesting result, and also of defining more precisely, by variations in cell dosage and refinements in technique, the requisite conditions for this to occur. Sixteen animals were given various dosages of epidermal cells suspended in citrate-saline, the volume injected being adjusted to about 5 ml. in every case. Unfortunately four of these animals (not entered in Table 1) became weak and lost consciousness within a few minutes of their injection. Complete and irreversible cessation of respiratory and cardiac function followed almost immediately. Trials exonerated both the citrate-saline and crude trypsin as possible causes of death—the latter substance being open to suspicion because of its use in separating the epidermis from the dermis. In the remaining 7 experiments, in which washed and resuspended cells were injected, no fatalities occurred. Whatever had caused the 4 deaths referred to was evidently present only in the medium in which the cells had been taken up and dissociated initially. Weisberger, Heinle, & Hannah (1949) found that sudden death in rabbits frequently followed their transfusion with leucocytes or leucocyte disintegration products, and obtained evidence suggesting that histamine might have been the causal agent. In the last 4 experiments set out in this table the animals were given a second injection of their donors' washed epidermal cells 12–15 days after the primary one; the grafting operation being carried out on the 22nd day after the primary injection as before.

Of the 19 animals that received injections comprising various dosages of their donors' epidermal cells, the lifetime of their skin homografts was significantly prolonged in 4. In one other, SR-223, which died under anaesthetic at the 9-day inspection, the condition of the grafts was such as to suggest that they would have survived until at least the 12th day. In none of the animals whose grafts had failed to show prolongation of their lifetime was there any evidence that breakdown was of the 'immune' type. Animals SR-205 and SR-254, whose grafts had shown prolongation of survival, were grafted a second time with their respective donors' skin on the 29th and 22nd days respectively after the first operation. In both animals the lifetime of these ‘second-set’ homografts was also significantly prolonged, though not to such an extent as that of their predecessors.

3. *Second series of experiments*

To take some cognizance of the possibility that the injection of *washed* cells might be less effective in bringing about the prolongation of graft survival than the injection of unwashed cells, despite the fact that less risk attached to it, the following regimen of injections was carried out in this series of experiments: The primary injection comprised washed cells, but the second injection, carried out 12–15 days later, although comprising mainly washed and resuspended cells, contained a known proportion of the initial cell suspension. In all animals the proportion of unwashed to washed cells was adjusted so as to be about 1 : 4. The results of the first 3 experiments in this series (see Table 2*a*) argued strongly in favour of the efficacy of this particular pre-treatment. It was following these successes that accurate cell counts were carried out on the cell suspensions before they were injected. Prolongation of the lifetime of the grafts was obtained in 9 of the 12 experiments carried out. It will be noted that 2 of the 3 animals which failed to show prolongation of the survival of their grafts had each received low dosages of cells at the primary injection, though an attempt had been made to compensate for this at the second injection. Of the 10 animals that received dosages of not less than 10 million cells intravenously, 9 showed prolongation of survival, which represents a high degree of uniformity in response. As with the first series of experiments, there was no suggestion that breakdown

TABLE 2*a*

The survival times of skin homografts transplanted to animals previously injected intravenously with their donors' epidermal cells. (Second series of experiments)

			No. of cells (millions) injected at		*Survival time of skin homografts (days)*	
Donor	*Recipient*	*Weight of recipient (kg.)*	*Primary injection*	*Secondary injection*	*First set*	*Second set*
SR-255	SR-261	3·5	10–12*	12–15*	20	7
SR-271	SR-272	2·5	10–12*	12–15*	16	8
SR-274	SR-275	2·5	12–15*	12–15*	16	8
SR-283	SR-284	2·5	10·0	10·0	19	12
SR-292	SR-293	2·5	11·3	10·4	19	13
SR-301	SR-302	3·6	6·7	13·0	7	—
SR-301	SR-303	3·7	6·7	13·0	9	—
SR-305	SR-306	2·2	10·5	11·0	>22	17
SR-309	SR-310	3·9	13·7	11·3	>22	10
SR-313	SR-314	2·4	11·0	11·3	8	—
SR-319	SR-320	2·2	10·4	11·5	>20	6
SR-323	SR-324	1·7	10·5	11·3	>20	8

* The actual number of cells has been estimated retrospectively in these experiments; see text.

of the 'immune' type took place in any animal whose grafts failed to show prolongation of survival.

All animals whose grafts had shown prolongation of survival were given a second set of their respective donors' skin grafts within 27–34 days after the first grafting operation. None of these 'second-set' grafts succumbed to a reaction of the 'immune' type; the grafts on two animals showed a degree of prolongation of survival that would have been adjudged significant had they been 'first-set' grafts (see figs. G, H, I, Plates 1 and 2). This argues strongly in favour of a persistence of the favourable conditions for skin homografts created in a host by its prior intravenous inoculation with the intended donor's epidermal cells.

TABLE 2*b*

The survival times of skin homografts transplanted to animals previously injected intravenously with their donors' washed *epidermal cells*

Donor	*Recipient*	*Weight of recipient (kg.)*	*No. of cells (millions) injected at*		*Survival time of skin homografts (days)*
			Primary injection	*Secondary injection*	
SR-329	SR-331	3·4	14·8	14·5	⩾6*
SR-339	SR-340	2·1	12·1	11·3	9
SG 339	SR-341	2·1	12·1	11·3	7
SR-342	SR-344	2·8	11·5	15·6	9

* Animal SR–331 died at the 6-day inspection when there was no indication that a specific reaction had commenced in the grafts.

The advantage of adding a portion of the initial cell suspension to the resuspended washed cells at the second injection is strongly suggested by the results of the 4 experiments set out in Table 2*b* in which animals were given two consecutive injections, each comprising washed cells only, before grafting. The dosages of cells administered were such that one would have expected prolongation of survival of the skin homografts as in the previous experiments. In 3 of the 4 recipients the grafts had succumbed to typical reactions by the 9th day. Unfortunately the remaining experiment was terminated by the death of the animal at the 6-day inspection when there was not the slightest indication of an incipient reaction in the grafts. They looked perfect and could confidently have been predicted to show survival until the 9th day at least had the animal lived.

4. *A note on the pattern of the reaction against homografts whose lifetime had been prolonged experimentally*

In the majority of those animals whose grafts showed prolongation of survival the reaction that eventually culminated in their total destruction followed, at least in broad outline, that against grafts transplanted to untreated animals, save that its onset was greatly delayed. Frequently, even by the 12th day, it would have been impossible, either on the basis of their outward appearance or by the histological examination of biopsy specimens, to differentiate such homografts from autografts of similar temporal standing. Epithelial outgrowth took place from their margins (Plate 1, fig. A) and by its coalescence with the outgrowth from neighbouring grafts the developing granulation tissue was completely resurfaced. Even when an incipient reaction could eventually be identified histologically—by lymphocytic infiltration of the graft dermis and usually by abnormal accumulations of oedema fluid in large sub-epidermal blisters or vesicles—the outlying epithelium continued to look perfectly normal. Frequently there was not the slightest suggestion that an incipient reaction was likewise developing in the granulation tissue lying immediately beneath it. In many cases the breakdown of the epidermis of the graft itself was practically complete before the reaction that developed against the outlying epithelium had progressed very far (Plate 2, fig. O). In untreated rabbits skin homografts are not normally outlived by epithelium which has migrated outwards from their margins (see Medawar, 1944).

The impression was gained that the reaction was frequently directed mainly against, or perhaps it was principally effective against, the dermis of the graft centres; the epidermis overlying it suffering only as a secondary consequence, and the outlying epithelium suffering least of all. Evidence consistent with this view was forthcoming from several animals whose grafts had almost completely succumbed to what appeared to be a normal and unmodified reaction by about the 9th day, and yet from whose margins there subsequently appeared small annuli of epithelial outgrowth that proliferated vigorously for many days thereafter, and even migrated centripetally to resurface the fibrotic remains of the graft dermis. In a few animals evidence was obtained that epithelial cells at the bases of some of the hair follicles outlived the superficial epidermis and proliferated vigorously to form a surface monolayer of epithelium, so replacing the original epidermis which had become necrotic as a result of the reaction taking place beneath it. Only in one animal was the modified reaction so delayed in its onset that new hairs actually formed and pierced the surface. The course of a typical modified homograft reaction that resulted in a survival time of greater than 22 days is illustrated in Plate 1, figs. B, C, D, E, and F.

PRELIMINARY ANALYSIS OF THE PHENOMENON

1. *Its specificity*

The experiments so far described simply establish that the expectation of survival of skin homografts may be significantly prolonged by the prior injection of an animal with a suspension of its intended donor's epidermal cells. It will now be shown that the injection of an animal with its *own* epidermal cells is without effect on its capacity to react against skin homografts. Four experiments were carried out in such a way that the fate of skin homografts in animals that had been injected with suspensions of their *own* epidermal cells could be compared with that of homografts transplanted to animals previously injected with cells derived from their intended graft donors. Each experiment made use of three rabbits chosen for their genetic disparity: D, DR, and R. A high dosage, washed, epidermal cell suspension prepared from animal DR was divided into two equal portions; one of these was injected intravenously into DR itself, and the other into animal R. These injections were repeated 15 days later; only this time the inocula were 'fortified' with some of the initial suspension, as described above. Nine days after the second injection skin homografts were transplanted according to the plan:

$$D \longrightarrow DR \longrightarrow R.$$

The results are set out in Table 3. Whereas the homografts transplanted to the animals pre-treated with their intended donors' cells all showed prolongations of their survival, the grafts transplanted to those animals which had received injections of their *own* cells succumbed to typical reactions within 9 days (see figs. M, N, Plate 2). The prolongation phenomenon is therefore not merely the non-specific outcome of pre-treatment of a host with epidermal cells as such, or some ingredient derived therefrom.

TABLE 3

Comparison of the lifetimes of skin homografts transplanted to animals previously injected intravenously with their own *epidermal cells with the lifetimes of homografts on recipients pre-treated with epidermal cells from their intended donors*

Donor-recipient (DR)	*Recipient (R)*	*No. of cells (millions) injected at*		*Survival time of skin homografts (days)*	
		Primary injection	*Secondary injection*	*(a) on DR*	*(b) on R*
SR-305	SR-306	10·5	11·0	8	>22
SR-309	SR-310	13·7	11·3	9	>22
SR-319	SR-320	10·4	11·5	8	>20
SR-323	SR-324	10·5	11·3	8	>20

The data relating to the recipients designated R in these experiments have been incorporated in Table 2*a*.

2. *The fate of the injected cells*

Knowledge of the fate of metastasizing tumour cells and of transfused leucocytes (see Weisberger *et al*., 1949, 1950; Osogoe, 1950) suggests that the injected epidermal cells probably accumulate and are removed from the blood-stream in the capillaries of the lungs. Even if dissociated Malpighian cells were capable of survival and proliferation in this anatomically unnatural environment, cells of homologous origin could scarcely be expected to outlive subsequent skin grafts from the same donor. Therefore the examination of the lungs of the majority of animals used in the experiments so far described would almost certainly have proved fruitless. However, the animals designated DR in the previous section were particularly suitable for this investigation since they had received injections of *autologous* cells on two separate occasions. They were destroyed 5 weeks after receiving the second injection of epidermal cells and a careful post-mortem examination was carried out. Not the slightest abnormality was revealed either by palpation or by dissection of the lungs with the aid of a lens, and the histological examination of representative portions of lung tissue revealed no clue as to the fate of the injected cells. Thus if the injected cells had been 'filtered off' in these organs there was no evidence that they had survived or proliferated. The only suggestion that the injected cells did in fact end up in the lungs was derived from one animal, in another series of experiments, which died 9 days after receiving a second injection of homologous epidermal cells (which had been prepared from pigmented skin). Histological examination of tissue removed from the periphery of the lungs revealed the presence of a few small granulomata in the centres of which were some scattered pigment granules. These might well have been the sites of breakdown of the epidermal cells.

3. *The importance of the potential viability of the injected cells*

In the experiments so far described the dissociated epidermal cells, whether washed or unwashed, were potentially viable at the time of injection. Trials had shown that if concentrated suspensions of washed or unwashed cells are disseminated or 'seeded' over extensive recipient areas on their donors' chests, of the type prepared to receive skin grafts (see above), they give rise to numerous foci of epithelium which, by fairly rapid growth, bring about the complete re-epithelialization of the areas. Experiments were therefore carried out to determine whether the prolongation of survival of skin homografts could be achieved by the prior injection of killed, but otherwise structurally and biochemically

TABLE 4

The survival times of skin homografts transplanted to animals previously injected intravenously with frozen and thawed suspensions of their donors' epidermal cells

Donor	*Recipient*	*Weight of recipient (kg.)*	*No. of cells (millions) injected at*		*Survival time of skin homografts (days)*
			Primary injection	*Secondary injection*	
SR-327	SR-328	2·0	10·8	13·5	7
SR-329	SR-330	2·8	11·2	13·7	9
SR-336	SR-337	2·4	10·0	15·3	12
SR-336	SR-338	3·2	10·0	15·3	6
SR-342	SR-343	2·7	11·5	15·6	7

intact, cells. The standard procedure of giving two injections, the second being fortified with unwashed cells, was carried out as hitherto. However, just before a suspension was injected it was frozen rapidly by immersing the thin-walled tube in which it was contained in liquid air (– 190° C.) for 10 minutes. It was then thawed out rapidly by immersion in a water-bath at 37° C. (Slow thawing would have been preferable, but this was found to result in the disintegration of a high proportion of the cells.) This freezing and thawing procedure is known to be lethal for dissociated epidermal cells (Billingham & Medawar, 1952).

These experiments are summarized in Table 4. The homografts on 4 of the 5 recipients broke down just as if they had received no pre-treatment. A survival time of 12 days for the grafts borne by SR-337 suggested that it might represent a significant prolongation. On the basis of these results it may be concluded that the injection of epidermal cell suspensions that have been killed by so conservative a treatment as freezing and thawing are far less effective, if they are effective at all, in prolonging the survival of skin homografts, than suspensions of *living* cells.

4. *The importance of the route of administration of the epidermal cells*

(*a*) *The efficacy of the intradermal route.* Five experiments were performed in which the recipients were given two intradermal injections—on days 0 and 12—of their intended donors' epidermal cells before receiving skin homografts on the 22nd day. Each injection, which was made into the shaved skin of the side of the animal's chest that was later to receive the skin grafts, was subdivided so that it raised upwards of a dozen scattered intradermal blebs. With the exception of animals SR-266 and SR-267, which received suspensions of unwashed epidermal cells, the remainder were injected with washed and resuspended cells.

TABLE 5

The effect of prior intradermal injections of homologous epidermal cells on the fate of skin homografts

Donor	Recipient	Weight of recipient (kg.)	No. of cells (millions) injected at: Primary injection	No. of cells (millions) injected at: Secondary injection	Survival time of skin homografts (days)
SR-265	SR-266*	2·0	12–15	10–12	6
SR-265	SR-267*	2·5	12–15	—	6
SR-325	SR-326	1·9	11·0	11·3	<6
SR-332	SR-333	2·7	14·0	14·0	6
SR-332	SR-334	3·4	14·0	14·0	6

* These animals received suspensions of unwashed cells, the number of cells injected being estimated retrospectively.

In all 5 animals breakdown of the grafts was of the 'immune' type (see fig. L, Plate 2). None of the grafts survived for longer than 6 days (see Table 5); in

SR-326 breakdown was clearly of long standing by the 6th day as evidenced by the histological appearance of the 6-day biopsy specimen. In the grafts borne by all animals except SR-266 some leucocytic infiltration of the graft dermis had taken place before disruption of the vessels intervened. In all cases the invading cell population, like that of the graft itself, was already dead by the 6th day.

(*b*) *The efficacy of the intraperitoneal route.* The frequent fatal consequences of injecting suspensions of unwashed epidermal cells intravenously have already been referred to. By introducing such suspensions intraperitoneally it was hoped that all risk would be eliminated and that the cells might rapidly enter the bloodstream as intraperitoneally injected erythrocytes are known to do (Courtice, Harding, & Steinbeck, 1953).

Three animals were accordingly given two consecutive intraperitoneal injections of suspensions of their donors' unwashed epidermal cells before they were test grafted. The results, summarized in Table 6, show that in none of these animals was there the slightest indication that the pre-treatment had influenced the fate of the homografts in any way.

TABLE 6

The effect of prior intraperitoneal injections of homologous epidermal cells on the fate of skin homografts

Donor	*Recipient*	*Weight of recipient (kg.)*	*No. of cells (millions) injected at*		*Survival time of homografts (days)*
			Primary injection	*Secondary injection*	
SR-345	SR-346	3·1	12·5	16·0	7
SR-345	SR-347	2·2	12·5	16·0	8
SR-348	SR-349	2·2	12·6	16·5	6

To prolong the life of skin homografts the administration of epidermal cell suspensions by the intravenous route appears to be obligatory.

5. *The effect of prior intradermal injections of epidermal cell washings on the fate of skin homografts*

It has already been mentioned that the supernatant left behind after spinning down the cells from an epidermal cell suspension is faintly opalescent and very rich in fine, almost sub-microscopic, particulate matter showing Brownian movement. This supernatant, when injected intravenously into a number of animals, was found to have the undesirable property of killing a proportion of them; in addition, there were good grounds for believing that it enhanced the efficacy of intravenously injected suspensions of washed epidermal cells in procuring the prolongation of survival of skin homografts.

A series of experiments was carried out to determine whether the prior intradermal administration of 'cell-free' epidermal cell washings would influence the fate of skin homografts transplanted subsequently from the donor of the cells. The 'washings' were obtained in the first instance simply by spinning down the cells from an initial volume of 5 ml. of citrate-saline containing 10–15 million cells. Microscopical examination of the resultant supernatant failed to reveal the presence of any cells. In the second series of experiments the supernatant was passed through a No. 3 porosity sintered glass filter before injection, to exclude all possibility of its containing cells. At each injection the material was subdivided so that at least a dozen intradermal blebs were raised in the shaved skin of the lateral thoracic wall of the recipient. All animals received two injections, on days 0 and 15, the skin homografts being transplanted on day 22.

TABLE 7

The effect of prior intradermal injections of epidermal cell washings on the fate of skin homografts

Donor	*Recipient*	*Whether washings passed through sintered glass filter*	*Survival time of skin homografts (days)*	*Type of reaction*
SR-255	SR-256	No	6	Immune
SR-255	SR-257	No	<6	Immune
SR-258	SR-259	No	<6	Immune
SR-258	SR-260	No	6	Immune
SR-274	SR-276	Yes	7	Normal
SR-277	SR-278	Yes	8	Normal
SR-279	SR-280	Yes	7	Normal
SR-292	SR-294	Yes	7	Normal
SR-309	SR-311	Yes	6	Immune

The results of the experiments are set out in Table 7. In all 4 animals that received the unfiltered material the skin grafts succumbed to reactions of the 'immune' type; in 2 of these animals breakdown was complete before the 6th day. But, of the 5 animals injected with material after its passage through a sintered glass filter, the grafts on only one showed decisive evidence of an 'immune' type of breakdown. Since no intact cells were demonstrable in the inocula received by the first 4 animals, and it has been shown that the intradermal injection of more than 11 million washed cells elicits an immune response comparable to that obtained in these animals, it seems unlikely that the discrepancy between the different results in the table can be ascribed to accidental inclusion of a few cells in the inocula not passed through the filter. The most likely explanation is that in the course of filtration much fine immunologically active particulate material was retained by the filters. If this explanation is accepted, then the evidence suggests very strongly that immunity to homologous skin can be elicited by material not containing living cells.

6. *The possibility of prolonging the life of skin homografts by the prior injection of cells not derived from skin*

Transplantation immunity is known not to be tissue specific; the prior grafting of a recipient with one type of tissue from a donor will prejudice the survival of a variety of other tissues subsequently transplanted from that donor (see Medawar, 1954). Therefore, it seemed of some importance to determine whether the lifetime of skin homografts could be prolonged by the prior intravenous injection of homologous cells not present in skin. Blood was the obvious source of cells for this purpose. Since, of the two distinct cell types present in blood, only the leucocytes are effective in eliciting transplantation immunity (Medawar, 1946; Billingham, Brent, & Medawar, 1956), particular attention has been paid to the actual numbers of these cells injected. The numbers of erythrocytes injected have been included for completeness' sake in the later experiments.

TABLE 8

The survival times of skin homografts transplanted to animals previously injected intravenously with their donors' blood

			Primary injection			*Secondary injection*			*Survival times of skin homografts (days)*	
Donor	*Recipient*	*Weight of recipient (kg.)*	*Vol. of blood injected (ml.)*	*No. of leucocytes (millions)*	*No. of erythrocytes* ($\times 10^9$)	*Vol. of blood injected (ml.)*	*No. of leucocytes (millions)*	*No. of erythrocytes* ($\times 10^9$)	*First set*	*Second set*
SR-285	SR-286	2·7	3	16	Not determined	3	15	Not known	>24	8
SR-285	SR-287	3·4	2	10·5	,,	2	10	,,	7	—
SR-288	SR-289	2·5	3	14·5	,,	3	27	12·6	11	—
SR-288	SR-290	3·6	5	24	,,	5	45	21	7	—
SR-288	SR-291	1·7	2	9·5	,,	2	18	8·4	9	—
SR-295	SR-296	2·0	2	17·5	,,	2·5	14·5	13	16	10
SR-295	SR-297	2·7	4	35·0	,,	5	29·0	26	8	—
SR-315	SR-316	3·0	2·5	20·5	14	2·8	20·0	11·8	9	—
SR-315	SR-317	3·2	2·5	20·5	14	2·8	20·0	11·8	9	—
SR-352	SR-353	2·6	2·5	14·0	13	3·1	14·0	14·3	15	—
SR-352	SR-354	2·4	3·0	17·0	15·5	3·8	17·0	17·5	9	—
SR-352	SR-355	3·0	3·5	19·5	18·0	4·3	19·5	19·8	8	—

Blood was obtained from the median ear artery in dilatation and immediately citrated to prevent clotting. The leucocyte content of all samples was determined after their citration, and in later experiments red-cell counts were also made. Each recipient was given two injections of its donor's blood, on days 0 and 12, skin homografts being transplanted on day 22 as hitherto. In the earlier experiments the volume of blood administered at both injections was kept approximately constant. However, as there was frequently a very great disparity between the actual numbers of leucocytes administered on the two occasions, in later experiments the volume of blood injected on the second occasion was adjusted so that the number of leucocytes it contained was similar to that injected previously. The 12 experiments carried out are summarized in Table 8. In 3 recipients a significant prolongation of the survival of the skin homografts was obtained (see Plate 2, figs. J and K). When 2 of these animals subsequently received a second set of their donors' skin grafts the latter did not succumb to reactions of the 'immune' type. In one other animal, SR-289, the pattern of the reaction against the grafts suggested very strongly that it had been modified by the pre-treatment. It may be significant that the 3 animals whose grafts showed a significant prolongation of survival had received comparable dosages of leucocytes, i.e. 14–17·5 million cells. This is comparable to the dosage of dissociated epidermal cells found to be effective. The effect of repeated massive intravenous injections of blood was investigated in 3 animals, SR-362, SR-367, and SR-369 (not included in Table 8). On days 0, 2, 4, 6, 8, and 10 each was given the equivalent of 12 ml. of citrated whole blood from its donor (making 72 ml. in all; and an aggregate leucocyte dosage of at least 700 million cells). It received grafts of its donor's skin on day 22. The fact that breakdown of the grafts borne by 2 of these animals was not complete until the 11th day, and in the third animal by the 9th day, suggests very strongly that their survival had actually been prolonged as a consequence of these prior massive intravenous injections. This implication is being investigated more fully.

TABLE 9

The survival times of skin homografts transplanted to animals previously injected intravenously with leucocyte concentrates of their donors' blood

			No. of leucocytes (millions) injected at		
Donor	*Recipient*	*Weight of recipient (kg.)*	*Primary injection*	*Secondary injection*	*Survival time of skin homografts (days)*
SR-356	SR-357	2·8	12	12	10*
SR-356	SR-358	3·25	10	10	7
SR-359	SR-360	2·1	11·4	11	8

* Modified reaction, presumably as a consequence of the pre-treatment.

Three animals were injected with concentrates of leucocytes from their donors' blood. These were prepared by centrifugation of the freshly drawn and citrated blood to obtain the buffy coat which was then resuspended in citrate-saline. These experiments are summarized in Table 9. Although a significant prolongation of the life of the homografts was not obtained in any of these animals, in SR-357 there was evidence that the reaction against its grafts had been modified as a result of the pre-treatment, the outlying epithelium outliving that of the graft centre by about 2 days (Plate 2, fig. O).

DISCUSSION AND CONCLUSIONS

It has been shown that a dosage of homologous, dissociated, viable epidermal cells which, if injected intradermally, will immunize a recipient against subsequent skin homografts, elicits no detectable immunity when it is administered by the intravenous route. Thus in this respect the behaviour of homologous epidermal cells is comparable with that of leucocytes (see Medawar, 1946). However, the intravenous injection of homologous epidermal cells is not without a specific immunological effect on the recipient, for its capacity to react against subsequent skin homografts from the donor of the cells may be modified or abrogated to an extent that increases the graft's expectation of survival by a factor of 2 or 3. In some animals this appears to be simply the outcome of a delay in the onset of what is otherwise a perfectly normal homograft reaction; but in others, however, the fundamental pattern of the histological reaction may be altered. In the latter case the reaction appears to be directed almost specifically against the dermal component of the homografts, being, at least for a time, without decisive effect against their epidermal component.

This induced modification of a host's normal resistance to grafts of homologous skin is of some duration; even after the breakdown of grafts of its donor's skin is eventually complete, subsequent transplants from that donor usually behave as if transplanted to an animal which had not previously received and reacted against skin homografts. But in some the survival time of these 'second-set' homografts, though less than that of their predecessors, was still significantly greater than the survival of homografts transplanted to untreated recipients. The weakness of the host's reaction against the epidermal component of skin homografts need not be ascribed to the fact that the pre-treatment comprised the injection of epidermal cells, for injections of homologous blood, whose cell population is entirely of mesenchymal origin, may have a similar result (Table 8). The abrogation of the host's resistance is therefore probably not tissue specific.

It has been found empirically that the prior injection of living homologous tissue cells can modify a host's response towards subsequent transplants, in graft form, of the same (genetically) living cellular antigen. A full causal analysis has yet to be undertaken. In the meantime it may not be out of place to consider likely explanations, or at least possible affinities, of the phenomenon in the light of our present knowledge.

1. Although they elicit no immune response—possibly because of their extreme physiological remoteness from the lymph-nodes (see Medawar, 1946)—the intravenously injected cells, or some derivatives from them, persist and absorb the 'protective' iso-antibodies later formed in response to the skin homografts, so exempting the latter from the maximum effect of the host's reaction. Most serious among several shortcomings of this explanation is its failure to take

into consideration the minute quantity of cells that is effective, for there is no evidence that they proliferate after injection.

2. The partial abrogation of the host's normal resistance to grafts of a donor's skin that results from its prior intravenous injection with homologous cells may be related to the following phenomena: (*a*) The enhancement of growth of certain homologous tumours subsequently inoculated into various rodents after their pre-treatment with frozen-thawed, lyophilized, heated or homogenized normal or malignant tissues having the same genetic constitution as the living tumour to be implanted (see Flexner & Jobling, 1907; Casey, 1934*a*, 1934*b*, 1941; Day *et al.*, 1954; and review by Snell, 1952). In all these studies, and in marked contrast to the present findings, the enhancement was obtained by the administration of *non-living* material by the *intraperitoneal* route; the amount of material injected being greatly in excess of that required in the present experiments. (*b*) The prolongation of survival of skin homografts in rabbits which Allen *et al.* (1952) believe may be brought about by treatment of the recipients with saline extracts of their intended donors' skins. Unfortunately no evidence was presented to show that this effect was, in fact, immunologically specific, and Billingham & Medawar (1953) were unable to reproduce the results claimed. In comparing these various phenomena it must be borne in mind that there are two criteria according to which the abrogation of a host's resistance to homologous tissue transplants may be appraised: either by challenging it with tumour material or with grafts of a normal tissue, such as skin. Students of transplantation immunity are agreed that the latter is by far the more exacting test, since a degree of impairment of a host's capacity for immunological response sufficient to enable a tumour implant to grow and eventually destroy its host may result in a reprieve for a skin homograft of no more than a few days from its normal fate.

Finally, there is one other phenomenon concerned with an altered response to heterologous antigens that may be formally related. Felton (1949) has demonstrated that the resistance of mice to subsequent inoculations with pneumococci may be abolished following their prior injection with a massive dose of the same type-specific pneumococcal polysaccharide. Morgan, Watson, & Cromartie (1952) have suggested that this phenomenon of 'immunological paralysis' may also occur in rabbits.

Although the phenomenon described in this paper has no immediate clinical bearing, it may indicate a possible line of approach towards a temporary solution of the clinical homograft problem; provided, of course, that the immunological consequence of introducing homologous cells intravenously in man is, at least in general outline, comparable to that in the rabbit. (The responses of animals of different species to antigens administered by any particular route may vary enormously—e.g. mice may be immunized against skin homografts by the prior *intravenous* injection of minute amounts of their donors' blood as Billingham, Brent, & Medawar (unpubl.) have found.) It has yet to be found whether a continuation of the 'homologous cell' therapy *after* the grafts have been transplanted will enhance their survival still further, and what is the minimum time that must be allowed to elapse between pre-treatment and skin grafting.

The great variability in the results obtained with blood-cells (see Table 8) as opposed to epidermal cells (see Table 2*a*) in prolonging the survival of skin homografts may be ascribable to variations in the population structure of the leucocytes. Irrespective of whether the number of cells injected was constant, the *proportions* of the various cell types present may have varied greatly from one experiment to another.

Reasons why the mammalian foetus, *qua homograft*, normally fails to provoke an immunity reaction from the mother have long been sought (see review by Medawar, 1953), and it is generally held that the most important single factor is its vascular isolation. Our present findings suggest that in rabbits the accidental leakage of foetal cells into the maternal blood-capillaries, far from compromising the well-being of the embryo, may even positively enhance its 'expectation of survival'.

One other finding that calls for special comment is the apparent antigenic effectiveness of cell-free epidermal cell washings. Attempts to elicit immunity to homologous skin with derivatives of skin not containing *living* homologous cells have been made hitherto, but without success (see Medawar, 1954).

SUMMARY

1. A study has been made of the effect of prior intravenous injections of living homologous dissociated epidermal cells and blood-cells on the survival of skin homografts subsequently transplanted from the cell donors in rabbits.

2. These treatments failed to elicit an immune response resulting in the accelerated breakdown of the skin homografts, even when a massive dosage of homologous blood was administered. On the contrary, in a high proportion of animals injected with their intended donors' epidermal cells it was found that the host's capacity to react against the skin homografts was abrogated to an extent that actually increased their survival by a factor of 2 or 3. This prolongation of survival is not merely the outcome of the non-specific treatment of a host with epidermal cells as such, since pre-treatment of animals with suspensions of their *own* epidermal cells was ineffective in prolonging the survival of homografts.

3. This modification in the host's response was not obtained by the administration of epidermal cell suspensions by other routes; when the cells were injected intradermally a state of immunity was elicited, and their intraperitoneal injection was without demonstrable effect.

4. The injection of *living* cells was found to be obligatory since cells killed by freezing and thawing before their injection had no effect on the course of the homograft reaction.

5. The injection of homologous blood gave results comparable with those

obtained with epidermal cell suspensions, although they were not as consistent.

6. Evidence is presented suggesting that a state of immunity towards grafts of homologous skin may be evoked by prior intradermal injections of epidermal cell washings not containing intact living cells.

7. Possible explanations and affinities of the induced abrogation of a host's immunological response to skin homografts are discussed, as well as its possible implications.

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REFERENCES

Allen, H. L., Williams, R. D., Lovingood, C. G., & Ellison, E. H. (1952). The effect of donor skin desensitization and ACTH on survival of skin homografts in rabbits. *Ann. Surg.* **135,** 239–44.

Amos, B. B., Gorer, P. A., Mikulska, B. M., Billingham, R. E., & Sparrow, E. M. (1954). An antibody response to skin homografts in mice. *Brit. J. exp. Path.* **35,** 203–8.

Billingham, R. E., Brent, L., & Medawar, P. B. (1956). *Phil. Trans. B.* (in press).

—— Krohn, P. L., & Medawar, P. B. (1951). Effect of locally applied cortisone acetate on survival of skin homografts in rabbits. *Brit. med. J.* **ii.** 1049–53.

—— & Medawar, P. B. (1951). Technique of free skin grafting in mammals. *J. exp. Biol.* **28,** 385–402.

—— —— (1952). The freezing, drying and storage of mammalian skin. *J. exp. Biol.* **29,** 454–68.

—— —— (1953). 'Desensitization' to skin homografts by injections of donor skin extracts. *Ann. Surg.* **137,** 444–9.

—— & Reynolds, J. (1952). Transplantation studies on pure epidermal epithelium and on epidermal cell suspensions. *Brit. J. plast. Surg.* **5,** 25–36.

—— & Sparrow, E. M. (1954). Studies on the nature of immunity to homologous grafted skin, with special reference to the use of pure epidermal grafts. *J. exp. Biol.* **31,** 16–39.

Casey, A. E. (1934*a*). Specificity of enhancing materials from mammalian tumors. *Proc. Soc. exp. Biol., N.Y.* **31,** 663–5.

—— (1934*b*). A persistent hypersusceptibility induced in rabbits with an homologous tumor material. *Proc. Soc. exp. Biol., N.Y.* **31,** 666–7.

—— (1941). Experiments with a material from the Brown–Pearce tumor. *Cancer Res.* **1,** 134–5.

Courtice, F. C., Harding, J., & Steinbeck, A. W. (1953). The removal of free red blood cells from the peritoneal cavity of animals. *Aust. J. exp. Biol.* **31,** 215–25.

Day, E. D., Kaliss, N., Aronson, A. I., Bryant, B. F., Friendly, D., Gabrielson, F. C., & Smith, P. M. (1954). Investigations of substances in mouse tissues inducing alteration of normal host-homograft relationships. *J. nat. Cancer Inst.* **15,** 145–59.

Felton, L. D. (1949). The significance of antigen in animal tissues. *J. Immunol.* **61,** 107–17.

Flexner, S., & Jobling, J. W. (1907). On the promoting influence of heated tumor emulsions on tumor growth. *Proc. Soc. exp. Biol., N.Y.* **4,** 156–7.

Heslop, R. W., Krohn, P. L., & Sparrow, E. M. (1954). The effect of pregnancy on the survival of skin homografts in rabbits. *J. Endocrinol.* **10,** 325–32.

MEDAWAR, P. B. (1944). The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat., Lond.* **78,** 176–9.

—— (1945). A second study of the behaviour and fate of skin homografts in rabbits. *J. Anat., Lond.* **79,** 157–76.

—— (1946). Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. exp. Path.* **27,** 15–24.

—— (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symposia of the Society for Experimental Biology*, **7,** 320–38.

—— (1954). General problems of immunity, in *Preservation and transplantation of normal tissues*: Ciba Foundation Symposium, ed. G. E. W. Wolstenholme and M. P. Cameron. Churchill: London.

MORGAN, P., WATSON, D. W., & CROMARTIE, W. J. (1952). Immunization of rabbits with Type II pneumococcal polysaccharide. *Proc. Soc. exp. Biol., N.Y.* **80,** 512–15.

OSOGOE, B. (1950). Transplantation of hematopoietic tissues into the circulating blood. *Anat. Rec.* **107,** 193–213.

SNELL, G. D. (1952). The immunogenetics of tumour transplantation. *Cancer Res.* **12,** 543–6.

WEISBERGER, A. S., HEINLE, R. W., & HANNAH, R. (1949). Transfusion of leukocytes and products of disintegrated leukocytes. *Proc. Soc. exp. Biol., N.Y.* **70,** 749–53.

—— —— STORAASLI, J. P., & HANNAH, R. (1950). Transfusion of leukocytes labelled with radioactive phosphorus. *J. clin. Invest.* **29,** 336–41.

EXPLANATION OF PLATES

PLATE 1

FIG. A. Showing 5 skin homografts 12 days after transplantation to an extensive recipient area prepared on the thorax of rabbit SR-293 which had received prior intravenous injections of its donor's cells. The grafts are ringed by broad annuli of keratinized epithelial outgrowth and there are still no outward indications that a specific reaction is in progress. ×1.

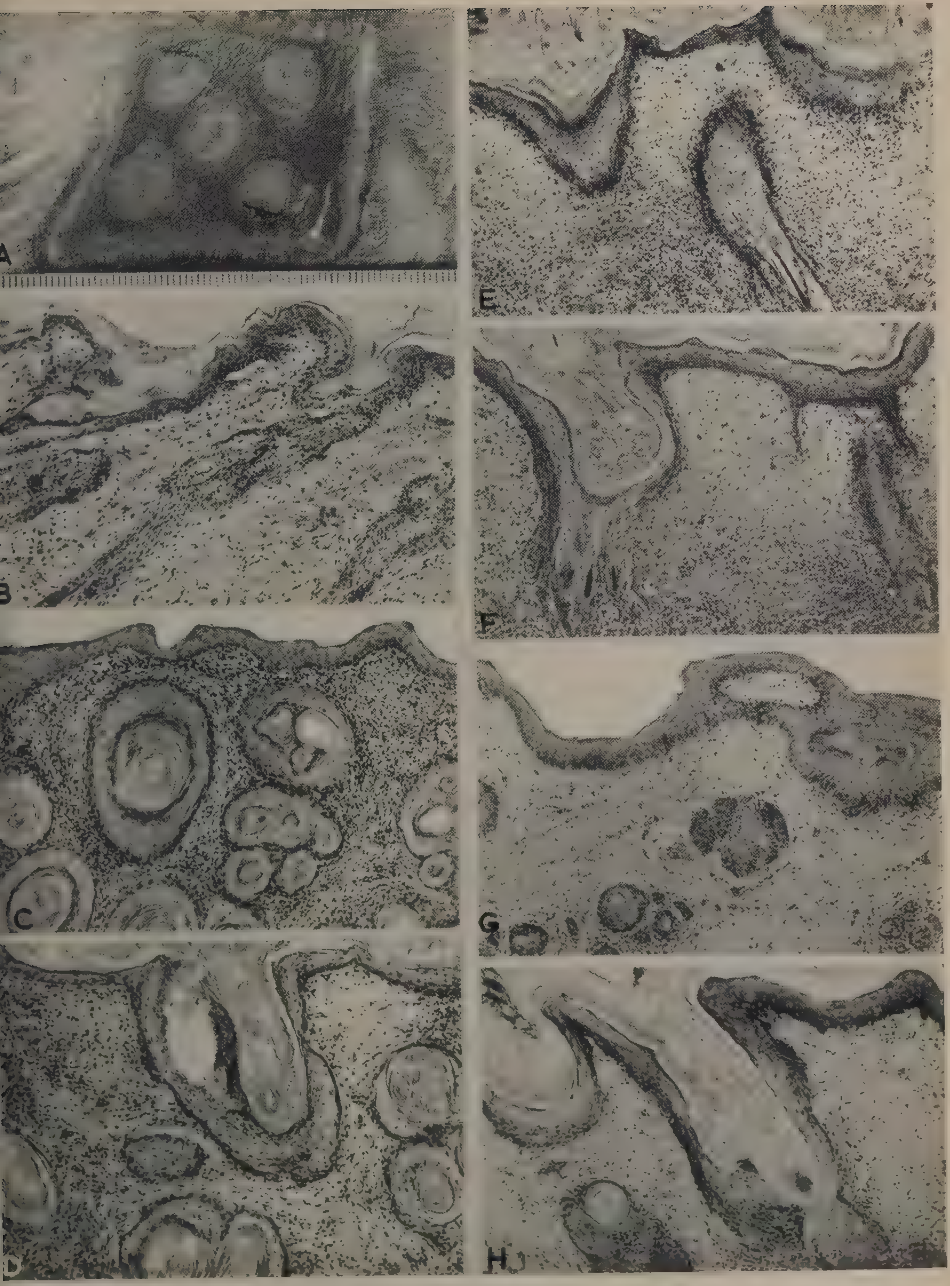
FIGS. B, C, D, E, and F. Transverse sections through biopsy specimens taken from the skin homografts on animal SR-310 on the 6th, 12th, 15th, 18th, and 22nd days respectively after transplantation. This animal received prior intravenous injections of its donor's epidermal cells. There is no evidence of an incipient reaction in the 6-day specimen which is autograft-like in all respects. In the 12-day specimen there is heavy infiltration of the graft dermis by leucocytes of native origin. In spite of chronic cellular infiltration and slight oedema of the dermis, illustrated by the figures, the animal's response has obviously been greatly modified since there is still a high degree of epithelial survival in the 22-day specimen. ×85.

FIGS. G, H, and I. Sections through specimens removed from the 'second set' skin homografts on rabbit SR-306, on the 10th, 12th, and 15th days after transplantation, whose response to previous homografts from the same donor had been considerably modified as a consequence of prior intravenous injections with its donor's epidermal cells. There is a very high degree of survival of the epithelium, which is hyperplastic, in all 3 specimens. This appearance is completely atypical of 'second set' homografts on untreated recipients and illustrates persistence of the induced modification of a host's response. ×85.

PLATE 2

FIGS. J and K. Sections through specimens, taken at 9 and 22 days respectively, from the skin homografts on animal SR-286 which had received prior intravenous injections of its intended donor's blood. Apart from a slight diffuse sprinkling of native leucocytes in the dermis of the 9-day graft, there is practically no suggestion of the onset of a specific reaction. The fact that there is still a high degree of epithelial survival in the 22-day specimen is strong evidence that the animal's response to its homografts has been greatly modified. ×85.

FIG. L. Section through a 6-day skin homograft transplanted to animal SR-333 which had previously been injected *intradermally* with its donor's epidermal cells. Breakdown of the graft is practically complete as a consequence of a reaction of the 'immune' type. Note the characteristic



R. E. BILLINGHAM & E. M. SPARROW

Plate 1

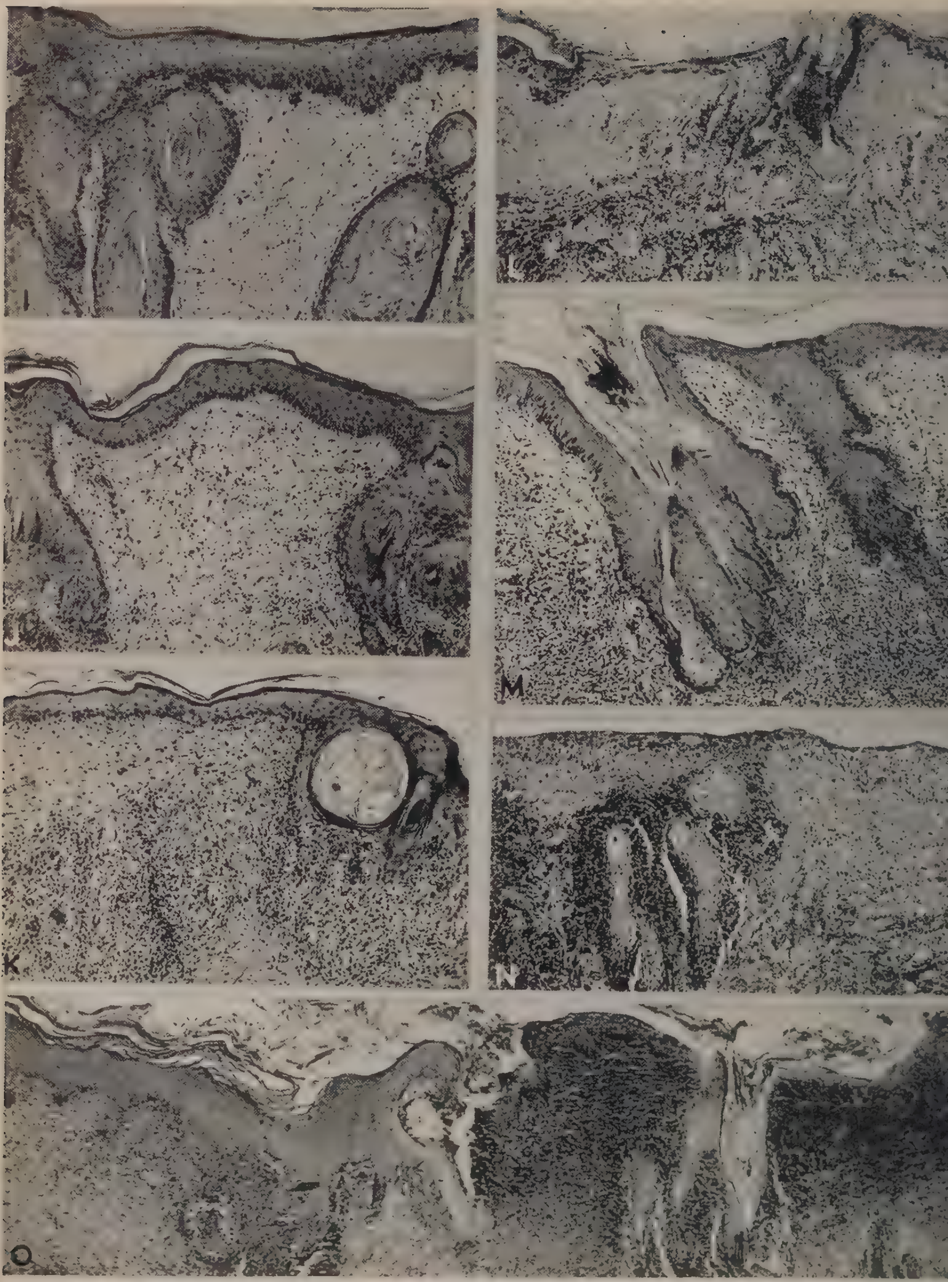


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Plate 2

absence of both oedema in the graft dermis and epidermal proliferation. Although there has been very slight leucocytic infiltration, the invading cells are dead and represented by chromatic droplets. ×85.

Figs. M and N. Sections through grafts of 6 and 9 days standing respectively, removed from animal SR-305 which had previously been injected intravenously with suspensions of its *own* epidermal cells. In the 6-day specimen a normal homograft reaction is far advanced as evidenced by the swelling of the dermis and heavy leucocytic infiltration. Breakdown of the graft is complete in the 9-day specimen. The invading cell population is dead and the epidermis, though anatomically intact, is completely necrotic. ×85.

Fig. O. Section through an 8-day skin homograft, including the actual graft centre and its epithelial outgrowth, removed from rabbit SR-357. This animal had received prior injections of its intended donor's leucocytes. Although the homologous epidermis only survived for 10 days, the course of the reaction was greatly modified. It can be seen that the graft centre has broken down completely and is necrotic, whereas there is still a very high degree of survival in the epithelium which had grown out from its margins over the native granulation tissue. ×85.

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On the Nature of Head Malformations Obtained by Centrifuging the Eggs of *Limnaea stagnalis*

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WITH TWO PLATES

INTRODUCTION

WHEN eggs of *Limnaea stagnalis* are treated with lithium chloride at early stages, characteristic aberrations of development are produced (Raven, 1942). On the one hand, vesicular or dumb-bell-shaped exogastrulae may develop; on the other hand, some of the embryos gastrulating normally show various malformations, especially of the head region, at later stages. The latter belong partly to the cyclocephalic series; but other malformations, such as microphthalmia, monophthalmia, eye reduplications, asymmetries of the head, and malformations of the mouth parts, may also be produced.

Raven (1949) studied the nature of the cyclocephalic head malformations (synophthalmia, cyclopia, anophthalmia, acephaly) produced by lithium. It appeared that these malformations are due to a suppression of the differentiation of mediodorsal parts of the head. In normal development the head is formed from two bilaterally situated anlagen, the cerebral plates, which each give rise to a cerebral ganglion, an eye, and a tentacle field. They are separated by a median band of seven large ciliated cells, the apical plate. Posteriorly, this is continuous with the head vesicle, a transverse girdle of twelve big swollen cells; anteriorly, the apical plate comes together above the mouth with the two halves of the velum, which border the cerebral plates ventrally and laterally, and merge with the lateral angles of the head vesicle posteriorly.

In cyclocephalic embryos produced by lithium the differentiation of the posterior part of the apical plate had in all cases been suppressed. In most cases the tentacle fields of both sides had fused in the midline. Very often the cerebral commissure was shortened, and the cerebral ganglia of both sides had fused to a common mass. The differentiation of the head vesicle, the velum, and the anterior part of the apical plate had been suppressed in a certain percentage of cases.

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In normal development the posterior four cells of the apical plate are derived from the cells immediately surrounding the animal pole of the egg (Holmes, 1900; Wierzejski, 1905). As the differentiation of this part was the most consistently suppressed in cyclocephalic embryos, and, in general, the effects of the treatment decreased with increasing distance from this point, it was concluded that these cyclocephalic malformations are due to a depressing influence of lithium on a gradient-field with its high point at the animal pole of the egg.

This hypothesis was corroborated by a study of the exogastrulae produced by lithium (Raven, 1952). A progressive series of suppression of animal differentiations was found in these exogastrulae. In some of them (type I), two fields of small-celled ectoderm, representing the cerebral plates, were surrounded by an 8-shaped area of big ectoderm cells, corresponding to apical plate, velum, and head vesicle. In type II the cerebral plates had fused to a single area of small-celled ectoderm, surrounded by a ring of big ectoderm cells. Finally, in type III the small-celled ectoderm of the animal hemisphere had been entirely suppressed.

The hypothesis that an animal gradient-field plays a role in the development of *Limnaea* received further support from the results of heat shock experiments. Visschedijk (1953) found that *Limnaea* eggs exposed to a heat shock at early stages yielded both exogastrulae and head malformations. A study of the head malformations produced in this way (Raven, De Roon, & Stadhouders, 1955) showed that they do not belong to the group of cyclocephalic monsters. The most characteristic malformation in these embryos consists in a reduplication of the posterior part of the apical plate, with formation of a dorsomedian tentacle field, which often gave rise to an eye or a tentacle. It was argued that this aberration of development can be explained in a satisfactory way by admitting that in these cases a *strengthening* of the animal gradient-field has taken place, which has led to a splitting of the field apex. In a way, therefore, this syndrome forms the reverse of the cyclocephalic malformations.

Exogastrulae and head malformations can also be produced in *Limnaea* by centrifuging the eggs at early cleavage stages. This was first shown by Raven & Van Egmond (1951). The highest number of abnormalities was produced by centrifuging at a late 4-cell stage (st. 14–15 according to Raven, 1946*a*); centrifuging at an early 4-cell or an early 8-cell stage was much less effective. The abnormal embryos showed various malformations; besides highly teratomorphic embryos, others were found in which local malformations of the head region were observed resembling those obtained after lithium treatment: displacements, reductions and reduplications of eyes, evaginations of the pharynx. But the head malformations were mostly combined with abnormalities of the shell, the foot, or other parts of the body.

These results were corroborated by those of Raven & Koevoets (1952). Both exogastrulae and head malformations were found after centrifuging. A combination of centrifugation and lithium treatment resulted in higher percentages of exogastrulae and head malformations than were produced by each of these treatments alone. No indications of a complex interaction between the influences of lithium and centrifugation were obtained.

Paris (1953) studied the exogastrulae produced by centrifuging. They resemble lithium exogastrulae both as regards their external shape and their histological structure. The same tissues in a comparable state of differentiation and with similar spatial relationships may be distinguished in both. However, in all exogastrulae produced by centrifuging which were investigated, two fields of small-celled ectoderm (cerebral plates) were present in the animal hemisphere. So they all belonged to type I, whereas the presence of types II and III could not be established. It was concluded from these observations that no suppression of animal differentiations takes place after centrifuging. Accordingly, it had to be admitted that the head malformations obtained after centrifuging the eggs are not due to a suppression of the animal gradient-field, but originate in another way than those produced by lithium treatment.

This supposition can only be tested by a study in sections of head malformations, produced by centrifugation.

MATERIAL AND METHODS

Egg-masses of *Limnaea stagnalis* were centrifuged at stage 14–15 (hence immediately prior to 3rd cleavage) for 10 minutes at a velocity of 2,500 r.p.m.; distance from the axis 13 cm., centrifugal pressure 925 g. After removal from the centrifuge the egg capsules were freed from the outer jelly, and laid out on an agar bottom in the usual way. They were cultured at 25° C. After reaching the 'hippo-stage' (cf. Raven, 1949), the embryos showing malformations of the head region were decapsulated and fixed in Bouin's fluid. They were embedded in paraffin, and cut in a transverse direction in serial sections of 7·5 μ thickness. The sections were stained with iron haematoxylin and erythrosin.

RESULTS

Nineteen embryos with malformations of the head region have been studied in sections. They show the following malformations:

Lateral reductions:	
reduction of eye	14
reduction of lateral tentacle	10
reduction of cerebral ganglion	3
Eye reduplications:	
unilateral	4
unilateral tripled	1
bilateral	1

Supernumerary structures:	
tentacle field	6
cerebral ganglion	6
eyes	6
statocyst	1
Various malformations:	
evagination of stomodaeum	2
splitting of foot	1

(*a*) *Lateral reductions*

In 12 embryos one of the eyes has been reduced; in 1 embryo the reduction concerns both eyes. In 9 cases no trace of this eye has been found; in 5 embryos one of the eyes is normally developed, the other is underdeveloped or rudimentary. In 7 cases the reduction concerns the left, in 7 cases the right side.

The eye reductions are often combined with other malformations of the head. Especially, the lateral tentacle of the same side is often reduced; in 4 cases it is lacking altogether, in 6 it is smaller than that of the other side. In 3 cases the cerebral ganglion, on the side where both the eye and the lateral tentacle are entirely lacking, is greatly reduced in size; in the other 10 embryos no difference in size between the cerebral ganglia of both sides could be observed.

In general the big-celled ectoderm of the head shows a normal differentiation in these embryos. The apical plate is present in all cases. The head vesicle is lacking in one embryo, but present in all other cases. In one embryo the velum is poorly differentiated. In the other cases it is well developed.

Table 1 summarizes the results in this group.

TABLE 1

Lateral reductions

No.	*Reduced side*	*Eye*	*Lateral tentacle*	*Cerebral ganglion*
36	left	red.	+	+
6	right	red.	red.	+
20	left	red.	red.	+
21	right	red.	red.	+
33	right	red.	red.	+
26	left	0	+	+
38	left	0	+	+
42	left	0	+	+
14	right	0	red.	+
44	right	0	red.	+
37	left	0	0	+
7	right	0	0	red.
34	right	0	0	red.
44	left	0	0	red.

+ = normal; red. = reduced; 0 = lacking.

(*b*) *Eye reduplications*

In 6 embryos eye reduplications have been found (the cases with multiple supernumerary eyes are not included in this number, cf. below). In 1 embryo the eyes of both sides are enlarged and have two cavities, each containing a lens. In all other respects the embryo is entirely normal. In 4 cases one of the eyes is reduplicated. In 2 of them this eye has two cavities each with a lens; in another embryo there are two contiguous eyes in the base of the tentacle; in the fourth embryo there is, besides a normal eye in the tentacle base, a second, more or less rudimentary eye ventral to the tentacle. Finally, in one case one eye is triplicated, containing three cavities each with a lens.

Of the 5 unilateral reduplications, 4 concern the left eye, 1 the right.

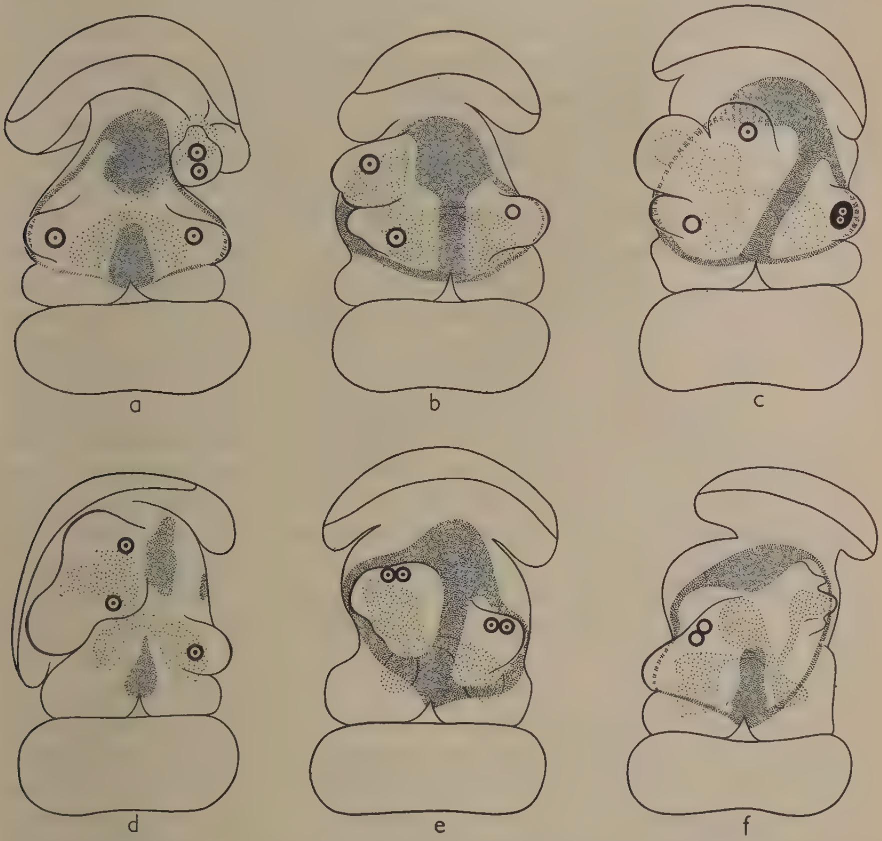
Although this malformation may be combined with various other abnormalities, in general the lateral tentacle and cerebral ganglion of the same side are normal. Only in one case the cerebral ganglion appears to be larger than normal; in the other embryos both cerebral ganglia are about equal in size. The apical plate, head vesicle, and velum are well developed in all cases.

(*c*) *Supernumerary structures*

In six embryos, in addition to the normal complement of head organs (which may, however, in part be more or less reduced), supernumerary structures have been developed. Together, they form a series of related malformations. In view of their importance for the interpretation of the centrifugation effects, these six cases will be described in detail.

No. 15. This embryo is for the greater part entirely normal (Text-fig. 1*a*). The intestinal sac, shell, and foot are well developed. The head is symmetrical, the eyes and tentacles show no deviations from a normal structure. The nervous system is well developed. The apical plate, head vesicle, and velum are well differentiated and show, in general, their normal relations. It is true that the apical plate does not extend to the head vesicle; both areas are separated by a short stretch of more or less indifferent epithelium. But this has nothing to do with a partial suppression of the apical plate, as occurs in cyclocephalic monsters; as a matter of fact, the normal number of apical plate cells is present. Such breaks in the continuity of the apical plate have also been met with in other cases (cf. below, and Raven, De Roon, & Stadhouders, 1955, embryo No. 5). Presumably, they occur in older embryos as a result of stretching of the dorsal part of the head and the 'neck' region.

The only abnormal feature of the embryo is represented by an outgrowth to the left of the head vesicle. Its dorsal part extends freely upwards (Plate 1, fig. A), whereas its base is connected with the left side of the 'neck' and with the left margin of the mantle fold. The left velum passes along the upper inner side of this outgrowth, so that it lies wholly outside the area surrounded by velum and head vesicle. It is covered by an epithelium which has the character of tentacle field epithelium. As a matter of fact, its dorsal part forms a distinct tentacle (fig. A). In its interior it contains two contiguous well-developed eyes, both having a lens (fig. A). In its basal part a well-differentiated ganglion has been formed (Plate 1, fig. B). This ganglion extends partly into the region of the mantle fold. It has remained in intimate connexion with the surface at the point where the outgrowth merges with the mantle fold epithelium. A distinct nerve fibre bundle extends from this ganglion forwards and to the right, crosses the midline some sections behind the cerebral commissure, and connects with the *right* cerebral ganglion.



TEXT-FIG. 1. Semi-diagrammatic drawings of head region (frontal view) of *a*, No. 15; *b*, No. 36; *c*, No. 33; *d*, No. 7; *e*, No. 34; *f*, No. 44. Dense stippling: big-celled ectoderm (apical plate, velum, and head vesicle). Wide stippling: cerebral ganglia.

No. 36. The intestinal sac and shell are rather abnormal in this case, but the head region and foot are fairly normal (Text-fig. 1*b*). The disposition of apical plate, head vesicle, and velum is the normal one (apart from an interruption in the right velum, cf. below). The lateral tentacles of both sides are well developed, but the left eye is reduced and does not contain a lens. The nervous system is intact, the cerebral ganglia of both sides are about equal in size.

Above and behind the right lateral tentacle and to the right of the head vesicle there is an outgrowth having the character of a supernumerary tentacle field. It is connected with a broad base in the region of the posterior part of the right velum, which is interrupted at this point. So it appears that the supernumerary tentacle field is situated just at the boundary between the pre-trochal and the post-trochal part of the ectoderm. It contains a well-developed supernumerary eye (Plate 1, fig. C) and a ganglion, which is connected with the right cerebral ganglion (Plate 1, fig. D). The overlying ectoderm has the structure of tentacle epithelium.

No. 33. The intestinal sac, shell, and foot are fairly normal. The apical plate, head vesicle, and velum show their normal relations (Text-fig. 1*c*). The left eye exhibits a beginning reduplication, as it contains two cavities each with a lens. On the contrary, the right eye is somewhat underdeveloped; it has about the normal size, but lacks a lens. The right lateral tentacle is somewhat reduced in size.

Above and behind the right lateral tentacle there is a bipartite outgrowth. It is covered by tentacle field epithelium. Internally it contains, besides a certain amount of mesenchyme, a ganglion (Plate 1, fig. E) which is also bipartite. Its median portion is connected, by means of a fibre tract, with the dorsal part of the right cerebral ganglion in the neighbourhood of the cerebral commissure (Plate 1, fig. F). Above this portion a well-differentiated supernumerary eye is found.

This outgrowth, which has the character of a supernumerary tentacle field, in this case is situated wholly inside the right velum, hence in the area of pre-trochal ectoderm.

No. 7. The intestinal sac, shell, and foot are almost normal. The head, however, shows a very abnormal disposition (Text-fig. 1*d*). The apical plate and head vesicle are present but are separated by a stretch of indifferent epithelium. As in the case of No. 15, this is probably due to the stretching of the head in this rather old embryo; the apical plate consists of its normal number of cells, some of which extend with long processes in the direction of the head vesicle. The differentiation of the velum is very poor; on the left, a few velum cells are found; the right half of the velum is unrecognizable or lacking altogether. The left eye, tentacle, and cerebral ganglion are well developed; on the right side, however, the lateral tentacle and eye are lacking in their normal location, and the cerebral ganglion is greatly reduced in size. Above and behind the region of the absent right tentacle, and to the right of the head vesicle, there is a large, more or less tripartite outgrowth (Plate 2, fig. G). The epithelium covering its lateral side has the character of tentacle field epithelium. On its anterior and upper side, however, there is an extended degeneration zone, where the epithelial cells are partly necrotic; this may be due to some external injury. The outgrowth contains a big, lobate ganglion. Its inner fibre mass is in broad connexion with the cerebral commissure (Plate 2, fig. G). Against the dorsal side of this ganglion two eyes are found. They are lying rather wide apart, one at the anterior and the other at the posterior end of the ganglion mass. Both eyes are well developed and contain a lens.

No. 34. At first sight the head of this embryo appears rather normal (Text-fig. 1*e*). The apical plate, head vesicle, and velum exhibit their normal relations. On both sides there are two contiguous and well-differentiated eyes. Those on the left are lying in the base of a normal lateral tentacle. On the right there is a similar outgrowth which lies, however, slightly more dorsal, and which contains the eyes. Study of the sections shows, however, that there are three cerebral ganglia. Two of them are lying in their normal location on either side of the buccal cavity and are connected by the cerebral commissure. The right is somewhat reduced in size, however, and is not accompanied by a lateral tentacle and eye. The third cerebral ganglion is situated more dorsally and connects with the cerebral commissure from the dorsal side (Plate 2, fig. H). The eyes and lateral tentacle of this side are related to this supernumerary ganglion. Apparently, therefore, in this case also a supernumerary cerebral plate has been formed which has to a large extent replaced the normal right cerebral plate and suppressed its differentiation except for a reduced cerebral ganglion. This case is therefore entirely comparable to the previous one.

No. 44. The intestinal sac and foot of this embryo are fairly normal, but the shell is small and flat and covers only the left side of the intestinal hump. The apical plate, head vesicle, and velum are present (Text-fig. 1*f*), but the apical plate and head vesicle are separated by an area of small-celled ectoderm, which at least partly resembles tentacle field epithelium. Apparently, unlike Nos. 15 and 7, the separation between the two is a primary one, and is not due to secondary effects of stretching. The normal number of apical plate cells is present, however.

On either side of the buccal cavity there is a cerebral ganglion in its normal location, but the left one is greatly reduced (Plate 2, fig. J). Both cerebral ganglia are united by a well-developed cerebral commissure (Plate 2, fig. K). The epithelium lateral to the right cerebral ganglion has the character of tentacle field, and two bulges separated by a groove represent the rudiments of lip tentacle and lateral tentacle (fig. J). But no eye is present in the latter. On the left the epithelium at this place is somewhat abnormal, irregular, and wrinkled; no tentacle rudiments are indicated here. However, more dorsally and posteriorly there is a three-lobed tentacle in a very abnormal position on the left side of the intestinal sac (fig. J). That this indeed represents a displaced lateral tentacle is indicated by the fact that the left velum passes along the outer side of its base in the usual manner (Text-fig. 1*f*). At the base of this tentacle there is a supernumerary cerebral ganglion (fig. J), which is connected by means of a fibre tract with the rudimentary left cerebral ganglion beside the buccal cavity. A second supernumerary cerebral ganglion is situated more anteriorly. It is connected through a strong fibre bundle with the left end of the cerebral commissure (fig. K); from this point, it extends in a dorsoposterior direction and obliquely to the right towards the surface, which it reaches slightly to the right of the anterior end of the head vesicle. Between this ganglion and the normal right cerebral ganglion, which is perhaps somewhat enlarged by addition of cells to its dorsal circumference, two rather well-differentiated but slightly reduced eyes without lenses are found (fig. K).

It is evident that this embryo is much more abnormal than the previous ones. It agrees with them, however, in the fact that supernumerary cerebral plate differentiations are present behind their normal location, with a simultaneous suppression of the differentiation of the normal eyes, tentacles, and cerebral ganglia.

Table 2 summarizes the results in this group.

TABLE 2

Embryos with supernumerary cerebral plate differentiations

	Left			*Right*			*Supernumerary structures*			
No.	*Eye*	*Tentacle*	*Cer. gangl.*	*Eye*	*Tentacle*	*Cer. gangl.*	*Side*	*Eyes*	*Tentacles*	*Cer. gangl.*
15	+	+	+	+	+	+	left	++	+	+
36	red.	+	+	+	+	+	right	+		+
33	++	+	+	red.	red.	+	right	+		+
7	+	+	+	0	0	red.	right	++		+
34	++	+	+	0	0	red.	right	++	+	+
44	0	0	red.	0	red.	+	both	++	+	++

+ = present; ++ = double; red. = reduced; 0 = lacking.

As stated above, these six embryos exhibit a regular series of related malformations. In all of them supernumerary cerebral plate differentiations (eyes, tentacles, cerebral ganglia) have been found in abnormal places, posterior to their normal locations. In No. 15 these supernumerary structures are situated wholly outside the area of pre-trochal ectoderm (cf. Raven, 1952) which constitutes the head proper. In the other embryos they are situated more and more near the normal tentacle fields. At the same time the normal cerebral plate differentiations on the side of the supernumerary structures are more and more suppressed.

(*d*) *Various malformations*

The malformations of the intestinal sac and shell, encountered in these embryos, will not be described in detail here. The same holds as regards the disturbance in the invagination of the stomodaeum, which has occurred in two of these embryos. This malformation and the considerable dislocation of head organs which it entails will be described in a subsequent paper. It is no characteristic effect of centrifugation, as it may also occur after lithium or heat shock treatment.

A peculiar malformation has been found in one embryo.

No. 42. The intestinal sac, the shell, and the head of this embryo appear normal apart from the fact that it is monophthalmic, the left eye being suppressed. The foot, however, is entirely split in two lateral halves. Both halves begin a few sections in front of the mouth and connect with the lower side of the lip folds bounding the mouth (Plate 2, fig. L). Posteriorly, the mouth is bordered by a protrusion, forming a kind of lower lip. It contains first the tongue, then the radular sac, and entirely separates the two halves of the foot (Plate 2, fig. M). The latter end already a considerable distance in front of the posterior boundary of the radular sac.

The cerebral ganglia are situated on either side of the mouth cavity, and connected by a well-developed cerebral commissure (fig. L). Each cerebral ganglion further connects through a cerebro-pedal connective with the pedal ganglion of its side. Each of the latter is situated in its own half of the foot, beside the radular sac (fig. M). The pedal ganglia show a normal differentiation, but there is no pedal commissure. Against the posterior side of each cerebro-pedal connective there is a well-developed statocyst (fig. M). Moreover, a supernumerary statocyst is found on the left side somewhat in front of the cerebro-pedal connective (fig. L).

DISCUSSION

1. This investigation has shown, in accordance with Raven & Van Egmond (1951) and Raven & Koevoets (1952), that head malformations may be obtained in *Limnaea* by centrifuging the eggs at a late 4-cell stage. Moreover, it confirms the conclusion drawn by Miss Paris (1953) from her study of exogastrulae produced by the same treatment, that these head malformations cannot be due to a suppression of the animal gradient-field of the egg, but originate in a different way than the cyclocephalic malformations produced by lithium treatment.

As a matter of fact, neither synophthalmic nor cyclopic embryos have been found among these head malformations produced by centrifuging. In no case had the differentiation of the posterior part of the apical plate been suppressed. It is true that in a few embryos (cf. Nos. 15 and 17, pp. 290 and 292) the continuity between apical plate and head vesicle had been broken, but this was probably due to secondary stretching of the head; the normal number of apical plate cells was present in these cases. The latter holds for No. 44 too, though in this case the break in continuity between apical plate and head vesicle may have occurred as a primary disturbance of head pattern in consequence of the treatment. Anyhow, in the latter embryo also there has occurred neither a distinct fusion of the tentacle fields of both sides nor a shortening of the cerebral commissure with fusion of the left and right cerebral ganglion to a common mass. We may conclude, therefore, that no typical cyclocephalic malformations have been produced by centrifuging the eggs. Apparently this treatment does not primarily act by depressing the animal gradient-field.

Neither do the head malformations obtained in this way resemble those characteristic aberrations produced by a heat shock, in which the posterior part of the apical plate is reduplicated, and a supernumerary median tentacle field is formed between both rows of apical plate cells (Raven, De Roon, & Stadhouders, 1955). In no case was such a splitting of the apical plate observed, and the supernumerary tentacle field structures found were always situated asymmetrically on one side. The hypothesis of a strengthening of the animal gradient-field by the treatment, therefore, which accounted for these heat shock effects, does not apply to these cases either.

2. The morphogenetic effects of centrifuging on the structure of the head may be arranged into the following categories: (1) lateral reductions, (2) eye reduplications, (3) formation of supernumerary cerebral plate differentiations, (4) disturbances of stomodaeum invagination.

The first group consists of embryos in which the structures derived from the cerebral plates (eyes, tentacles, cerebral ganglia) are reduced on one side of the head. This malformation is not a typical centrifugation effect, for monophthalmic and microphthalmic embryos may also be formed after lithium or heat shock treatment.

However, while the eye reductions following a heat shock at the 2- to 4-cell stage are located on the right side in a large majority of the cases (Raven, De Roon, & Stadhouders, 1955), whereas they concern mostly the left side after lithium treatment (Raven, 1942), the unilateral reductions after centrifuging at the 4-cell stage are equally distributed between both sides. This may perhaps be explained in the following way. Both exposure to lithium and to a heat shock are general treatments, in which all parts of the egg (or, in the case of lithium, at least all parts of the egg surface) are subjected to the same external agent. Intrinsic differences in susceptibility between both sides, bound to the left-right asymmetry of the egg, may influence the result of the treatment. In centrifuging, however, the intervention itself is directional, as the displacement of egg substances takes place along the axis of centrifugation, which will, as a rule, deviate from the egg axis. If we assume that the direction of this deviation is arbitrary, then we may expect that the morphogenetic effects of centrifuging will be distributed at random over both sides of the body.

Table 1 (p. 289) shows another interesting relation. Not only does the frequency of lateral reductions decrease in the order eye–tentacle–cerebral ganglion, but (1) in no case is the tentacle reduced unless the eye of the same side is reduced or lacking, (2) in no case is the tentacle lacking unless the eye is lacking, (3) in no case is the cerebral ganglion reduced unless both eye and tentacle are lacking. It appears, therefore, that these three organs only become reduced in this sequence. This conclusion can only be accepted with some caution, however. The embryos to be studied were selected on account of their externally visible deviations, especially of eye development; isolated reductions of cerebral ganglia might easily have escaped detection. This group of embryos therefore forms no unbiased sample of cases. On the other hand, this sequence fits in with other observations in *Limnaea*, so that we may accept it provisionally as a rule of cerebral plate reduction. We may recall in this context that we have come to the conclusion in a previous paper (Raven, De Roon, & Stadhouders, 1955) that the eyes represent the highest gradient level among the organs deriving from the cerebral plates. In accordance with Child's views it is easily understood that they are the first organs to be suppressed by adverse influences on the differentiation of the cerebral plates. Following this line of argument, it may perhaps be assumed that the lateral tentacle corresponds to a lower, the cerebral ganglion to a still lower gradient level within the cerebral plate area.

3. Eye reduplications not only occur after centrifuging, but also after lithium treatment and heat shock. Apparently this is another malformation which may be quite easily produced by slight disturbances in the processes laying down the pattern of head organs. In our previous paper (Raven, De Roon, & Stadhouders, 1955) we have suggested that eye reduplication may occur in consequence of an excess of determining factors in the cerebral plate area. In that case, such an excess of determining factors was supposed to be due to a general rise in the strength of the animal gradient-field, especially in its lower levels. In our present experiments there are no indications of such an increase in the strength of the gradient-field. It must be especially emphasized that our embryos with eye reduplications, contrary to those obtained by heat shock, have in all cases a well-developed velum.

4. The third group of malformations represent the most characteristic effect of centrifugation, in so far as no similar deviations of development have been encountered thus far after other treatments. They consist of *the formation of supernumerary cerebral plate differentiations in abnormal places*. In its most typical form, a complete half of a head, consisting of an (often reduplicated) eye, a lateral tentacle, and a cerebral ganglion, has been formed. In some cases a tentacle of normal shape is lacking, but then it is represented by more or less blunt projections covered with tentacle field epithelium. However, a well-differentiated cerebral ganglion and at least one eye are present in all six embryos.

This supernumerary cerebral plate may be variously located, though in all cases it is lying behind the normal ones. It may lie entirely outside the area surrounded by velum and head vesicle, hence in the region of the post-trochal ectoderm (cf. Raven, 1952). In this case the differentiation of the 'host' cerebral plates is normal. In other cases, however, the supernumerary cerebral plate is situated nearer the normal ones, either on the boundary of or completely within the pre-trochal region. As it approaches one of the 'host' cerebral plates, the differentiation of the latter is more and more suppressed; and, finally, the supernumerary plate may almost altogether supersede and replace one of the normal cerebral plates, so that only the characteristic anomaly in the topography of the cerebral ganglia bears witness to the process that has taken place.

These centrifugation effects, with their production of isolated organ complexes beside and more or less independently of the normal pattern of differentiation, but interacting secondarily with the latter, bear an unmistakable resemblance to the results of Pasteels (1953 *a* and *b*, 1954) with centrifugation of blastulae and gastrulae of amphibians. Although the process of determination in the two groups is very different in nature, one cannot help suspecting that there must be some fundamental similarity in the processes underlying these effects.

5. When we compare the developmental aberrations produced by lithium, heat shock, and centrifuging, respectively, it appears that there are a number of malformations which they have in common. Exogastrulation may occur after all three treatments. The same holds for the lateral reductions, the eye reduplications, and the disturbances in the invagination of the stomodaeum. However, in addition to this after each of these three treatments there is a characteristic syndrome of malformations, which is (as far as our present knowledge goes) restricted to this treatment only. In the case of lithium, this is the group of cyclocephalic malformations (Raven, 1949). The characteristic syndrome of heat shock treatment is represented by the median reduplications with splitting of the posterior part of the apical plate (Raven, De Roon, & Stadhouders, 1955). Finally, the typical centrifugation effects consist in the formation of a supernumerary cerebral plate. It is clear that for the explanation of the effects of the treatments the latter malformations are of particular importance.

In order to explain the typical malformations obtained by lithium and heat shock treatment, respectively, we have put forward the hypothesis of the existence of a gradient-field with high point at the animal pole of the egg, which is responsible for the determination of organs in the animal hemisphere, especially for the development of the pattern of the head. The cyclocephalic malformations, on the one hand, and the median reduplications, on the other, have been interpreted in terms of an influence of the treatment on the strength of this field; a weakening of the field causes, according to this view, a suppression of the differentiation of mediodorsal head structures, whereas a strengthening of the field leads to their reduplication. In this way it was possible to bring the two groups of malformations under a common denominator.

When we now consider the malformations which have been described above as the most characteristic effects of centrifuging, it appears that this kind of explanation is not applicable here. It is true that the formation of an isolated cerebral plate in foreign ectoderm might in certain circumstances be interpreted as being due to a local increase in field strength, whereby the cells at this place reach the level corresponding to cerebral plate differentiation. However, one might expect in this case that at least in some of these embryos the level of apical plate differentiation would be attained at such a spot; and, at any rate, that the area of increased field strength would pass gradually into its surroundings, so that the isolated cerebral plate would be surrounded by the differentiation corresponding to a lower field level, i.e. velum. Neither of these expectations is realized, however. On the contrary, one gets the impression that *the normal pattern of head organs* (viz. two bilaterally situated cerebral plates, surrounded by the 8-shaped area of apical plate, head vesicle, and velum) *is not fundamentally disturbed in these embryos* (cf. Text-fig. 1), and that *the supernumerary cerebral plate is merely superimposed upon the normal pattern of the head.*

6. We have, therefore, to look for another explanation of these consequences of centrifugation, and it may be profitable to take into account the direct effects of this treatment. Under the influence of centrifugal force, the egg substances are stratified according to their specific weights. This applies, at least, to the more fluid inner cytoplasm. There are indications, however, that the more solid cortical layer of the egg, which forms the substrate of the factors controlling the polarity and, probably, the symmetry of the egg, is not displaced by moderate centrifuging. So, after centrifuging immediately after oviposition, the polar bodies are formed, presumably, at the original animal pole of the egg, and the animal pole plasm forms at its normal location (Raven & Bretschneider, 1942; Raven, 1945; Raven & Brunnekreeft, 1951).

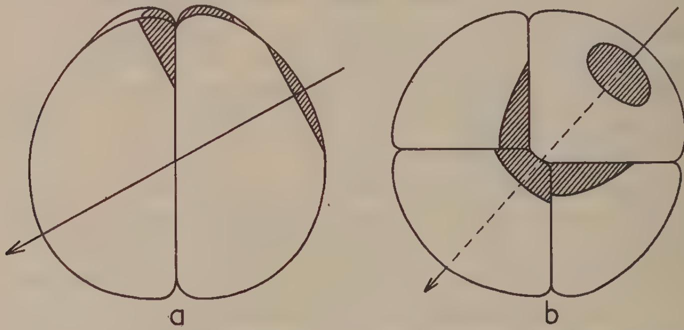
The substances displaced during centrifuging do not remain indefinitely in their new positions; they tend to redisperse through the egg in the hours following upon the treatment, and to assume their normal positions with respect to the axes of the egg (Raven & Bretschneider, 1942; Raven, 1946*b*). However, a complete recovery of the normal distribution of substances does not occur for some time, and, depending on the time and circumstances of the treatment, more or less distinct traces of the disturbances produced may remain visible for a long time (Raven, 1946*b*; Raven & Brunnekreeft, 1951). In particular, after centrifuging at an early moment after oviposition, a substance closely resembling the animal pole plasm may collect beneath the cortex at those places where formerly the hyaloplasm zone of the centrifuged egg was situated (Raven & Brunnekreeft).

In general, we may expect therefore that centrifuging will interfere with the processes of ooplasmic segregation. It is significant, in this respect, that the effects of centrifuging at a late 4-cell stage (st. 14–15) are much more pronounced than those of a treatment at earlier or later stages (Raven & Van Egmond, 1951). As a matter of fact, at this stage very important processes of ooplasmic segregation are taking place, the subcortical protoplasm concentrating towards the animal side of the cells and there uniting with the animal pole plasm to a common mass of dense protoplasm, which passes at the 3rd cleavage for the greater part into the micromeres (Raven, 1946*a*).

It may be remembered, further, that it is just at this stage that centrifugation took place in the present experiments. There are good grounds for the assumption, therefore, that *the effects of this treatment have to be explained as consequences of a disturbance of the ooplasmic segregation taking place at early cleavage stages.*

7. As stated above, one gets the impression that in general the pattern of head organs is not fundamentally disturbed in these embryos. It appears, therefore, that *centrifugation does not directly affect the animal gradient-field* lying at the basis of this pattern. This may be explained by the assumption that this gradient-field is located, at least at early cleavage stages, in the relatively immovable cortical layer.

On the other hand, the formation of a supernumerary cerebral plate must be connected with the disturbances of ooplasmic segregation brought about by the treatment. The most simple explanation appears to be that *cerebral plate differentiation is dependent on the presence of a morphogenic substance* which is displaced by centrifugal force. In normal development this substance is concentrated in two bilaterally situated areas on either side of the animal pole. Centrifugation at the late 4-cell stage leads to the accumulation of part of this substance at a single spot at some distance from the animal pole, whereas the rest is normally located.



TEXT-FIG. 2. Diagram of the influence of oblique centrifugal pressure on localization of light substances at the 4-cell stage. *a*, from the side; *b*, from above.

Text-fig. 2 may help to visualize such an explanation. It has been shown that, if *Limnaea* eggs are centrifuged during the uncleaved stage, the axis of centrifugation may make any angle with the egg axis; as a rule, however, its centripetal end is situated in the animal half of the egg (Raven & Brunnekreeft, 1951). If we assume this to be true also when the eggs are centrifuged at the 4-cell stage; if we further admit that the direction in which the axis of centrifugation deviates from the egg axis is arbitrary; then it is clear that the centripetal end of the former will, as a rule, be situated somewhere in the animal half of one of the four cleavage cells (Text-fig. 2*a*). In this cell a morphogenic substance which is thrown centripetally will be heaped up at some distance from the animal pole, whereas it is accumulated in the neighbourhood of this pole in the other three cells (Text-fig. 2*b*).

Of course, it is not certain that this explanation is correct in every detail. It may suffice, however, to give an example of the type of interpretation that may cover the facts. In particular, such an explanation may account for the fact that an isolated cerebral plate may be formed in foreign parts, while at the same time the normal cerebral plates of the embryo are more or less completely developed.

8. We have come to the conclusion, therefore, that the differentiation of the structures of the cerebral plates (eyes, lateral tentacles, cerebral ganglia) is dependent on the presence of a 'cerebral plate substance', which can be displaced by centrifugal force at an early cleavage stage. On the other hand, previous experiments have shown that these differentiations are also related, in one way or another, to the animal gradient-field. As a matter of fact, the disturbance of the pattern of the head brought about by a weakening or strengthening, respectively, of the animal gradient-field, involves a medial displacement of these organs, on the one hand, or the production of a supernumerary median field of cerebral plate differentiations, on the other. Moreover, indications have been obtained that in the subdivision of each cerebral plate into its component organs, field phenomena also play a part (cf. above, p. 297, and Raven, De Roon, & Stadhouders, 1955).

Since both a morphogenic substance and a gradient-field appear to play a part, therefore, in the differentiation of these organs, some interaction or co-operation between the two has to be assumed. One might think, for instance, that in normal development *the localization of the 'cerebral plate substance' occurs under the influence of the animal gradient-field located in the cortex.* The latter would form, so to speak, a system of co-ordinates in relation to which the arrangement of morphogenic substances takes place through attractive and repulsive actions between the cortex and the components of the inner protoplasm. We have been able to show, as a matter of fact, that the localization of the animal pole plasm in *Limnaea* must be due to such local attractions by the cortex (Raven, 1945; Raven & Brunnekreeft, 1951). It might be assumed that the 'cerebral plate substance' normally tends to accumulate beneath those parts of the cortex where the field factors have a certain value. A weakening or strengthening of the field, such as may occur after lithium and heat shock treatment, respectively, leads to a distortion of the 'system of co-ordinates', and hence to an abnormal localization of the cerebral plate substance. But the latter may also be brought about in a purely mechanical way by centrifugal force.

This interpretation of the facts accounts, therefore, in a satisfactory way for the results obtained after various treatments. Of course, other hypotheses on the interaction between morphogenic substance and gradient-field are conceivable. There is no sense, however, in speculating more fully on various possibilities, as it is too early to come to a decision as yet. We shall have to await further data to get a clearer insight into these problems.

SUMMARY

1. Eggs of *Limnaea stagnalis* were centrifuged at a late 4-cell stage for 10 minutes at a centrifugal pressure of 925 × gravity. The head malformations produced by this treatment were studied.

2. No cyclocephalic malformations were obtained. Apparently centrifuging does not produce a depression of the animal gradient-field.

3. No median reduplications with a splitting of the apical plate were found. Hence centrifuging does not cause a strengthening of the animal gradient-field either.

4. Lateral reductions of eyes, tentacles, and cerebral ganglia were obtained in a large percentage of the cases. They were equally distributed between both sides of the head. The frequency of lateral reductions decreases in the order: eye, tentacle, cerebral ganglion.

5. Lateral eye reduplications occurred in a certain number of cases. They were not accompanied by a reduction of the velum.

6. A complete splitting of the foot in two lateral halves was found in one case.

7. The most characteristic effect of centrifugation consists of the formation of a supernumerary cerebral plate in an abnormal place. This may lie wholly in the post-trochal or in the pre-trochal region, or on the boundary between the two.

8. As the supernumerary cerebral plate approaches one of the 'host' cerebral plates, the differentiation of the latter is more and more suppressed. Finally, the supernumerary plate may almost altogether supersede and replace one of the normal cerebral plates.

9. The formation of a supernumerary cerebral plate cannot be explained by a disturbance of the animal gradient-field. The supernumerary plate appears to be superimposed upon a relatively undisturbed pattern of head organs.

10. It is concluded that the normal pattern of head organs is controlled by a gradient-field located at early cleavage stages in the relatively immovable cortical layer. The differentiation of the cerebral plate, on the other hand, is dependent on a morphogenic substance which may be displaced by centrifugal force.

11. In normal development, some kind of interaction between morphogenic substance and gradient-field takes place. The most simple hypothesis appears to be that the localization of the 'cerebral plate substance' during ooplasmic segregation is controlled by the cortical gradient-field.

REFERENCES

Holmes, S. J. (1900). The early development of *Planorbis*. *J. Morph.* **16,** 369–458.

Paris, A. J. (1953). Histological investigations on exogastrulae of *Limnaea stagnalis* obtained by centrifuging the eggs. *Proc. Acad. Sci. Amst.* **C 56,** 406–8.

Pasteels, J. (1953*a*). Les effets de la centrifugation sur la blastula et la jeune gastrula des Amphibiens. I. Mécanisme de la formation des organes secondaires aux dépens de l'ectoblaste. *J. Embryol. exp. Morph.* **1,** 5–24.

—— (1953*b*). Les effets de la centrifugation sur la blastula et la jeune gastrula des Amphibiens. II. Étude comparative de la sensibilité en fonction des stades et des espèces. *J. Embryol. exp. Morph.* **1,** 125–45.

—— (1954). Les effets de la centrifugation sur la blastula et la jeune gastrula des Amphibiens. III. Interactions entre ébauches primaires et secondaires. IV. Discussion générale et conclusions. *J. Embryol. exp. Morph.* **2,** 122–48.

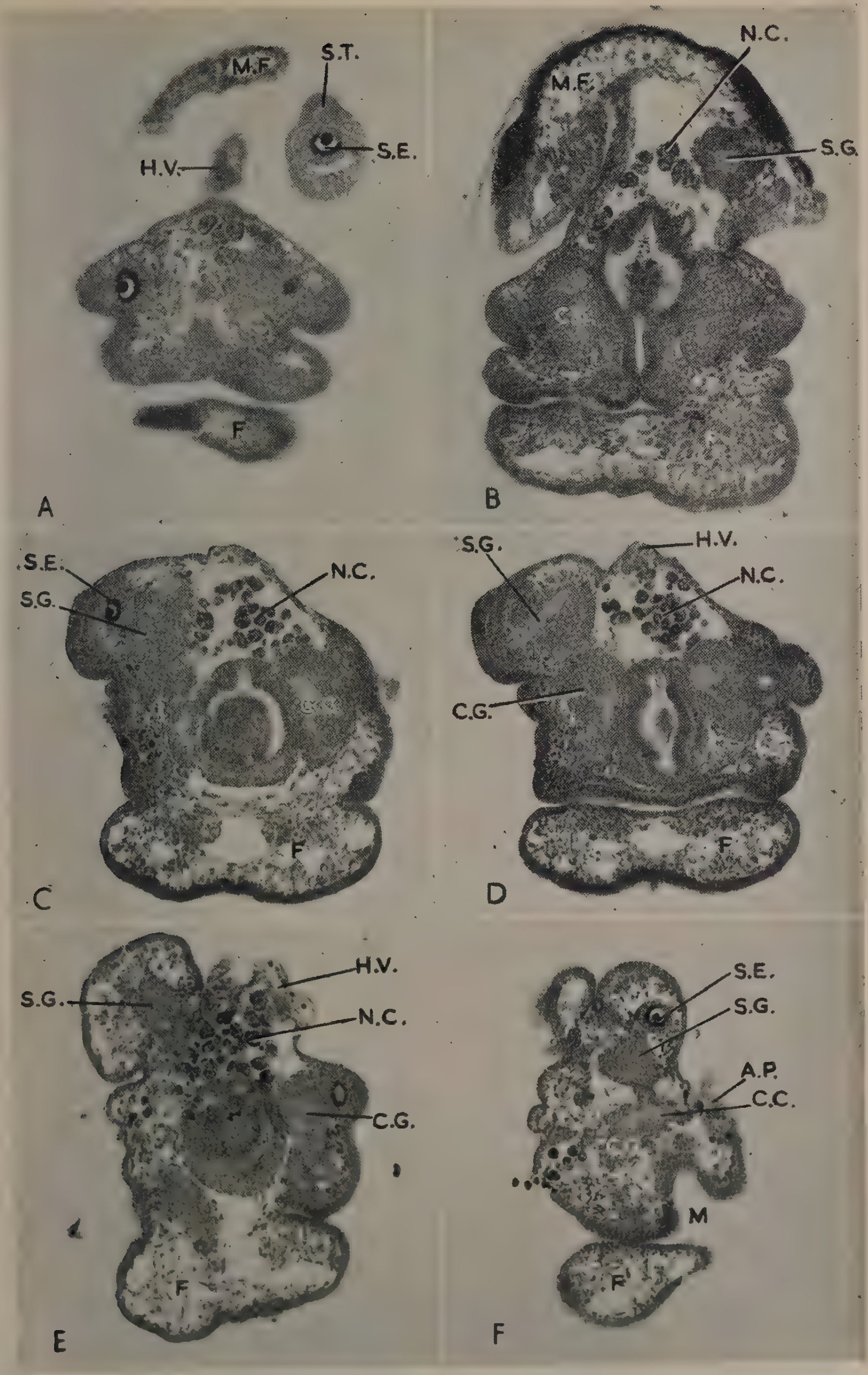


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Plate 1

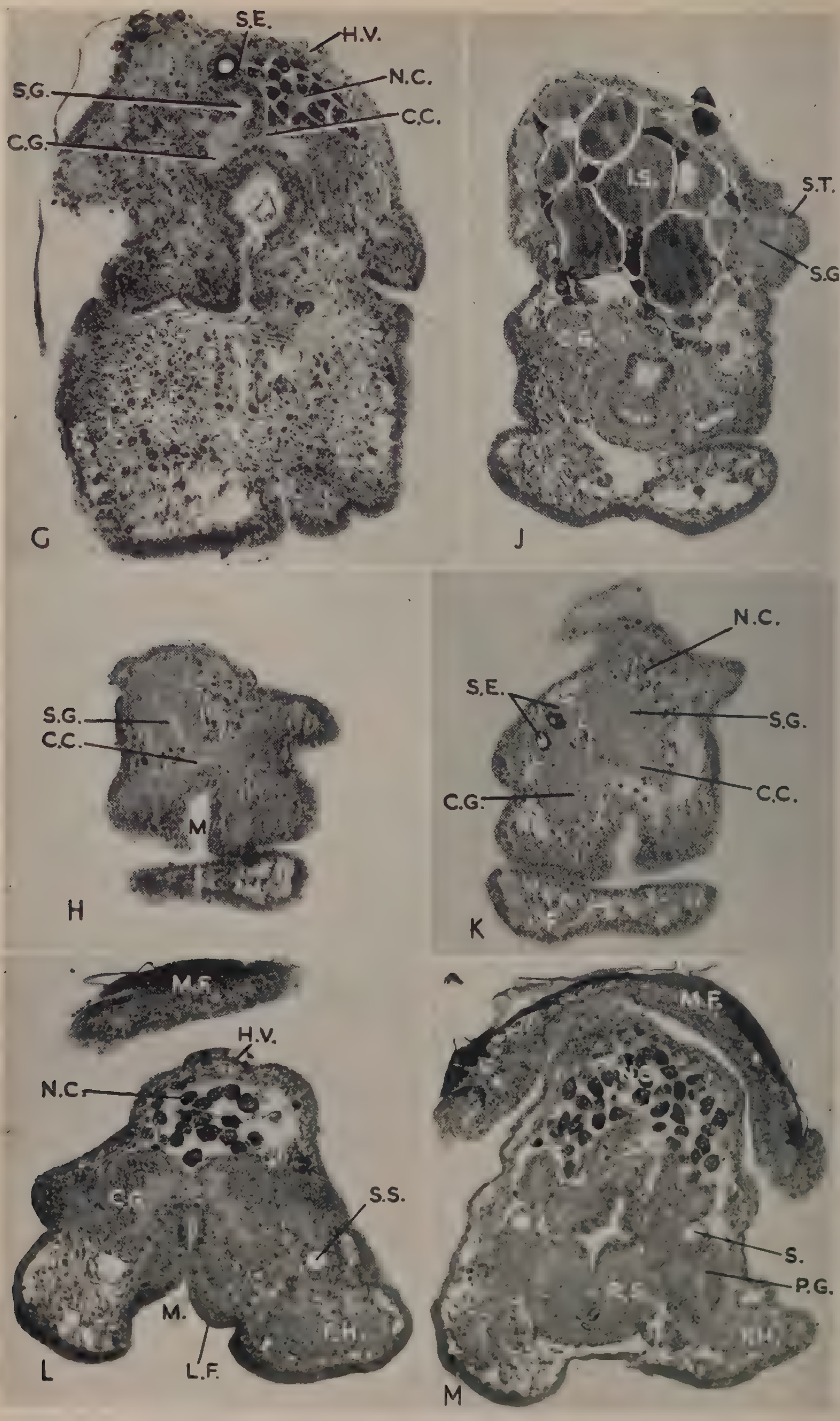

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Plate 2

RAVEN, CHR. P. (1942). The influence of lithium upon the development of the pond snail, *Limnaea stagnalis* L. *Proc. Acad. Sci. Amst.* **45,** 856–60.

—— (1945). The development of the egg of *Limnaea stagnalis* L. from oviposition till first cleavage. *Arch. néerl. Zool.* **7,** 91–121.

—— (1946*a*). The development of the egg of *Limnaea stagnalis* L. from the first cleavage till the trochophore stage, with special reference to its 'chemical embryology'. *Arch. néerl. Zool.* **7,** 353–434.

—— (1946*b*). The distribution of substances in eggs of *Limnaea stagnalis* L. centrifuged immediately before cleavage. *Arch. néerl. Zool.* **7,** 496–506.

—— (1949). On the structure of cyclopic, synophthalmic and anophthalmic embryos, obtained by the action of lithium in *Limnaea stagnalis. Arch. néerl. Zool.* **8,** 323–52.

—— (1952). Morphogenesis in *Limnaea stagnalis* and its disturbance by lithium. *J. exp. Zool.* **121,** 1–78.

—— & BRETSCHNEIDER, L. H. (1942). The effect of centrifugal force upon the eggs of *Limnaea stagnalis* L. *Arch. néerl. Zool.* **6,** 255–78.

—— & BRUNNEKREEFT, F. (1951). The formation of the animal pole plasm in centrifuged eggs of *Limnaea stagnalis* L. *Proc. Acad. Sci. Amst.* **C 54,** 440–50.

—— & VAN EGMOND, M. TH. C. (1951). Centrifuging the eggs of *Limnaea* round about the third cleavage. *Proc. Acad. Sci. Amst.* **C 54,** 325–31.

—— & KOEVOETS, TH. C. M. (1952). Combined effects of lithium and centrifuging on the eggs of *Limnaea. Proc. Acad. Sci. Amst.* **C 55,** 697–700.

—— DE ROON, A. C., & STADHOUDERS, A. M. (1955). Morphogenetic effects of a heat shock on the eggs of *Limnaea stagnalis. J. Embryol. exp. Morph.* **3,** 142–59.

VISSCHEDIJK, A. H. J. (1953). The effect of a heat shock on morphogenesis in *Limnaea stagnalis. Proc. Acad. Sci. Amst.* **C 56,** 590–7.

WIERZEJSKI, A. (1905). Embryologie von *Physa fontinalis* L. *Z. wiss. Zool.* **83,** 502–706.

EXPLANATION OF PLATES

All figures are transverse sections of the head region; magnification 155 ×.

Abbreviations: A.P. apical plate; C.C. cerebral commissure; C.G. cerebral ganglion; F, foot; F.H. foot half; H.V. head vesicle; I.S. intestinal sac; L.F. lip fold; M. mouth; M.F. mantle fold; N.C. nuchal cells; P.G. pedal ganglion; R.S. radular sac; S. statocyst; S.E. supernumerary eye; S.G. supernumerary ganglion; S.S. supernumerary statocyst; S.T. supernumerary tentacle.

PLATE 1

FIG. A. Embryo No. 15. Supernumerary cerebral plate structures (eye, tentacle) in outgrowth on left side of head vesicle.

FIG. B. Embryo No. 15. Supernumerary ganglion in connexion with mantle fold.

FIG. C. Embryo No. 36. Supernumerary cerebral plate structures (eye, ganglion) above right tentacle region.

FIG. D. Embryo No. 36. Connexion of supernumerary ganglion with right cerebral ganglion.

FIG. E. Embryo No. 33. Outgrowth with supernumerary ganglion above right tentacle region.

FIG. F. Embryo No. 33. Supernumerary ganglion connects with dorsal part of right cerebral ganglion. Supernumerary eye.

PLATE 2

FIG. G. Embryo No. 7. Supernumerary cerebral plate structures (eye, ganglion) above right tentacle region. Supernumerary ganglion in connexion with cerebral commissure.

FIG. H. Embryo No. 34. Supernumerary ganglion in connexion with cerebral commissure.

FIG. J. Embryo No. 44. Supernumerary cerebral plate structures (tentacle, ganglion) on left side of intestinal sac.

FIG. K. Embryo No. 44. Supernumerary ganglion connected with cerebral commissure. Supernumerary eyes.

FIG. L. Embryo No. 42. Section through the mouth. Anterior part of split foot.

FIG. M. Embryo No. 42. Section through radular sac. Posterior part of split foot.

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